Effects of Roux-en-Y Gastric Bypass Surgery on Energy Expenditure and Bone Metabolism in Rats

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1. Summary

The dramatic and progressive increase in worldwide obesity during the past years puts an enormous financial strain on national healthcare systems. Despite the continuous and tremendous effort to find pharmacological treatment options for morbid obesity, bariatric surgery remains the most effective long-term treatment. Roux-en-Y gastric bypass (RYGB) is considered the gold standard of bariatric procedures, although the mechanisms by which the anatomical rearrangement of the intestinal tract leads to sustained weight loss and a rapid decrease in comorbidities are still mostly unknown. Contrary to the traditional classification of RYGB as a restrictive and malabsorptive procedure, these factors seem to play a minor role. Increasing evidence suggests that changes in gut hormone levels, such as glucagon-like peptide-1 (GLP-1), and potentially in neuroendocrine signaling may account for the majority of the effects.

Increasing evidence suggests that one major side effect of RYGB surgery is a decrease in bone density. Vitamin D and calcium malabsorption with consequential secondary hyperparathyroidism have been suggested as potential causes, but the exact mechanisms are still unclear. In a longitudinal study in rats, we showed that bone mineral density decreased early after RYGB surgery and coincieded with intestinal calcium malabsorption. However, this seemed to be independent of vitamin D malabsorption, since increased vitamin D activation compensated for lower total vitamin D levels, and PTH levels were not elevated in RYGB rats. Although intestinal calcium absorption normalized between two and seven weeks after surgery, there was no restoration of bone mass. We identified chronic metabolic acidosis, which was associated with increased lactate levels and increased urinary calcium loss, as a potential mechanism underlying inhibited bone mass restoration. The RYGB-induced changes in bone metabolism occurred independent of weight loss, since they were not found in a sham-operated control group that was body weight-matched to the RYGB rats by food restriction.

Previous studies have shown that the compensatory decrease in energy expenditure in response to body weight loss is attenuated in RYGB rats. Since increased GLP-1 levels contribute to the reduced caloric intake after RYGB surgery, we hypothesized that they may also be involved in the reported alterations in energy expenditure. We therefore investigated the effects of acute peripheral GLP-1 agonism and antagonism on energy expenditure in RYGB and sham-operated control rats; however, we did not find any effect of either treatment on energy expenditure. In contrast, GLP-1 agonism decreased food intake more in RYGB than in sham rats, and GLP-1 antagonism significantly increased food intake in RYGB, but not in sham rats. This indicates that RYGB rats are more sensitive to the satiating effect of both endogenous GLP-1 and an exogenous GLP-1 agonist.

One important factor that has to be considered when measuring energy expenditure is body composition. Since fat is metabolically less active than lean tissue, differences in the fat to lean mass proportion between groups could lead to differences in energy expenditure. By computed tomography analysis, we were able to show that body composition did not differ between RYGB rats and sham operated rats that were food restricted to match the body weight of RYGB rats. We thereby confirmed that weight loss after RYGB surgery and by caloric restriction led to the same changes in body composition, which suggests that the reported alterations in energy expenditure In RYGB rats are not caused by differences in body composition.

The thermoneutral zone, i.e. the ambient temperature at which energy expenditure is lowest because there is no need for thermogenesis, is higher in rodents than in humans. This means that if energy expenditure in rodents is measured at room temperature, the animals may be under moderate cold stress leading to increased energy expenditure due to adaptive thermogenesis. We speculated that the reported alterations in energy expenditure of RYGB rats could be caused by an upward shift in the thermoneutral zone and therefore a higher requirement for thermogenesis at lower temperatures. However, we found no difference in the thermoneutral zone of RYGB compared to ad libitum fed sham operated rats and showed that the RYGB-induced changes in energy expenditure persisted at thermoneutrality.

In conclusion, we confirmed in our rat model that RYGB surgery leads to bone loss, potentially caused by intestinal calcium malabsorption. However, this seemed to be independent of vitamin D malabsorption or secondary hyperparathyroidism; but metabolic acidosis may play a role in the inhibition of bone mass restoration after normalization of calcium absorption.
We also confirmed that the compensatory decrease in energy expenditure after weight loss is attenuated by RYGB surgery and showed that this effect is not caused by differences in body composition or the thermoneutral zone of RYGB rats. Acute modulation of GLP-1 signaling did not influence the RYGB-induced alterations in energy expenditure, suggesting that other factors than increased GLP-1 levels underlie these findings.

2. Zusammenfassung

Die weltweit ansteigende Prävalenz von Obesitas und ihren Folgeerkrankungen führt zu einer massiven finanziellen Belastung der Gesundheitssysteme. Die zur Zeit einzig wirksame Therapie krankhafter Fettleibigkeit stellt die bariatrische Chirurgie dar, da die verfügbaren pharmakologischen Optionen entweder keinen ausreichenden und dauerhaften Gewichtsverlust erzielen oder zu schwerwiegenden Nebeneffekten führen. Der Roux-en-Y-Magenbypass (RYMB) ist der Goldstandard der bariatrischen Eingriffe, obwohl die genauen Mechanismen, welche zum Gewichtsverlust und zu einer schnellen Besserung der Begleiterkrankungen führen, noch nicht genau bekannt sind. Im Gegensatz zur ursprünglichen Idee, dass die Einschränkung der Magenkapazität zu einer verminderten Nahrungsaufnahme führt und dass gleichzeitig die Nährstoffabsorption im Darm durch die veränderte Anatomie reduziert wird, haben neuere Studien gezeigt dass diese Faktoren nur eine untergeordnete Rolle spielen. Hingegen scheinen eine erhöhte Sekretion gastrointestinaler Sättigungshormone wie zum Beispiel Glucagon-like Peptide-1 (GLP-1) sowie möglicherweise Veränderungen der neuroendokrinen Signalübertragung zu den positiven Effekten des RYMB beizutragen.

Immer mehr Studien deuten darauf hin, dass eine reduzierte Knochendichte als negative Begleiterscheinung des RYMB auftreten kann. Als mögliche Ursachen wurden Vitamin D- und Kalziummalabsorption mit daraus folgendem sekundärem Hyperparathyroidismus vorgeschlagen, die genauen Mechanismen sind jedoch ungeklärt. In einer Longitudinalstudie in Ratten konnten wir zeigen, dass die Knochen-Mineraldichte bereits kurz nach der RYMB-Operation abnahm. Dies fiel zeitlich zusammen mit intestinaler Kalziummalabsorption, schien jedoch unabhängig von einer Vitamin D-Malabsorption zu sein. Obwohl die Gesamtwerte von Vitamin D in RYMB-Ratten reduziert waren, war das aktive Vtamin D erhöht; die Parathormonwerte waren unverändert. Die intestinale Kalziumabsorption normalisierte sich zwar zwischen zwei und sieben Wochen nach der Operation, die Knochenmasse blieb jedoch reduziert. Wir konnten chronische metabolische Azidose im Zusammenhang mit erhöhten Laktatwerten und erhöhter renaler Kalziumausscheidung als mögliche Ursache für den fehlenden Knochenwiederaufbau identifizieren. Die Veränderungen im Knochenmetabolismus nach RYMB-Operation waren unabhängig vom Gewichtsverlust, da sie in einer restrikitv gefütterten Kontrollgruppe mit gleichem Gewichtsverlust nicht auftraten.

Frühere Studien haben gezeigt, dass die kompensatorische Herunterregulierung des Energieumsatzes als Folge von Gewichtsverlust in RYMB-Ratten abgeschwächt ist. Da die erhöhte GLP-1 Sekretion nach RYMB-Operation zur reduzierten Energiaufnahme beiträgt, könnte sie auch einen Einfluss auf den Energieumsatz haben. Wir untersuchten deshalb die Auswirkungen von akuten peripheren Injektionen eines GLP-1 Agonisten und eines GLP-1 Antagonisten auf den

Energieumsatz und die Energieaufnahme von RYMB und Sham-operierten Ratten. Beide Substanzen hatten keinen Effekt auf den Energieumsatz; der Effekt auf die Energieaufnahme war jedoch stärker in den RYMB-Ratten als in den Sham-operierten Ratten. Dies deutet darauf hin, dass das Sättigungspotential sowohl von endogenem GLP-1 als auch eines exogen verabreichten GLP-1 Agonisten in RYMB-Ratten erhöht ist.

Ein wichtiger Faktor bei der Bestimmung des Energieumsatzes ist die Körperzusammensetzung, beziehungsweise das Verhältnis zwischen Fett- und Magermasse. Da Fett eine wesentlich geringere metabolische Aktivität als andere Gewebe hat, können Unterschiede im Körperfettanteil bei gleichem Körpergewicht zu einem unterschiedlichen Energieumsatz führen. Wir konnten jedoch mittels computertomographischer Analyse zeigen, dass keine Unterschiede im Körperfettanteil von RYMB-Ratten und einer restriktiv gefütterten Kontrollgruppe mit gleichem Gewichtsverlust bestanden. Dies bestätigt, dass die beschriebenen Veränderungen im Energieumsatz von RYMB-Ratten nicht durch ein unterschiedliches Verhältnis zwischen Fett- und Magermasse verursacht wurden.

Die Umgebungstemperatur, bei welcher keine zusätzliche Wärmeerzeugung zur Erhaltung der Körpertemperatur notwendig ist, wird als thermoneutrale Zone bezeichnet. Diese ist für Nagetiere höher als für Menschen, weshalb Ratten und Mäuse bei Raumtemperatur unter einem gewissen Kältestress stehen und aktiv Thermogenese betreiben, was den Energieumsatz erhöht. Wir spekulierten, dass die beschriebenen Veränderungen im Energieumsatz von RYMB-Ratten durch eine Erhöhung der thermoneutralen Zone und dadurch erhöhte Thermogenese bei Raumtemperatur verursacht wurden; fanden jedoch keinen Unterschied in der thermoneutralen Zone im Vergleich zu Sham-operierten und ad libitum gefütterten Ratten. Zusätzlich konnten wir zeigen, dass die Veränderungen auch bei Thermoneutralität bestehen blieben.

Zusammenfassend konnten wir in unserem Rattenmodell bestätigen, dass die RYMB-Operation zu Knochenverlust führt, möglicherweise durch Kalziummalabsorption im Darm. Dies scheint unabhängig von Vitamin D-Malabsorption oder sekundärem Hyperparathyroidismus zu sein, jedoch könnte eine chronische metabolische Azidose den Knochenwiederaufbau nach Normalisierung der Kalziumabsorption hemmen.
Wir haben zusätzlich bestätigt, dass die kompensatorische Herunterregulierung des Energieumsatzes als Folge von Gewichtsverlust in RYMB-Ratten abgeschwächt ist und konnten zeigen, dass dies nicht durch einen reduzierten Körperfettanteil oder eine Erhöhung der thermoneutralen Zone verursacht wird. Ebenfalls scheinen die erhöhten GLP-1 Werte nach RYMB-Operation keine direkte Rolle bei den beschriebenen Veränderungen zu spielen.

3. Introduction

3.1. Energy homeostasis

Maintenance of energy homeostasis, i.e. the balance between energy intake and energy expenditure, is crucial to keep body weight stable. The modern world is confronted with a wide increase in the prevalence of obesity and of related disorders such as diabetes mellitus, atherosclerosis and hypertension; together, they express the dramatic consequences of imbalances in energy homeostasis.

3.1.1. Energy intake

The hormonal system controlling energy intake consists of two main categories, satiation signals and adiposity signals, which are sensed and integrated by the central nervous system (CNS).

Satiation signals consist of peptides released from the gastrointestinal tract in response to food intake. The variable distribution of different cell types secreting specific peptides within the gastrointestinal tract, combined with specific secretion stimuli for each peptide, provide the body with accurate information about the amount and composition of ingested nutrients and thus facilitate appropriate digestion and processing of nutrients. In addition, these hormones exert acute effects on food intake and thereby contribute to meal termination. If administered shortly before a meal, they dose-dependently reduce meal size; while blocking endogenous signaling with specific receptor antagonists before a meal increases meal size. Such effects have been shown, among others, for cholecystokinin (CCK),[1-3] amylin,[4-6] GLP-1,[7,8] glucagon[9] and peptide YY (PYY)[8,10,11]; although it often proves difficult to block actions of endogenous peptides.[7,12] Since the magnitude of the postprandial gut hormone response depends on the amount of calories ingested,[13,14] satiation signals play a crucial role in the short-term control of energy intake.

Adiposity signals on the other hand are hormones secreted in proportion to body fat and therefore provide long-term information about the energetic status of the body. The best-characterized adiposity signals are leptin, secreted directly from white adipocytes,[15-17] and insulin, which is secreted from pancreatic beta cells.[18,19] It has recently become clear that, in contrast to the traditional view, adipose tissue is not just a metabolically inactive organ for fat storage, but also an endocrine organ secreting diverse hormones and cytokines that are involved in the control of metabolic function. However, none of these newly discovered adipose-derived signals have been identified as classical adiposity signals yet.[20]

After their release, satiation signals either reach their central site of action via the bloodstream or act in a paracrine manner on local nerve endings. The β-cell derived hormone amylin for example is co-secreted with insulin into the blood and acts directly on the area postrema in the hindbrain,[21] which is accessible to blood-borne signals due to the lack of a functional blood brain barrier.[22] Other

gastrointestinal hormones, including CCK,[23] GLP-1[24] and PYY[10,24] mediate their effects at least partly via receptors on local afferent fibers of the vagus nerve. Although there may also be a non-vagal component to the signaling of these peptides, the integrity of the vagus seems to be crucial for their effects. The plasma half-life of satiation signals is generally very short, reflecting their role in the short-term control of food intake. However, the half-life of peptides acting on vagal afferents generally seems to be even shorter compared to the ones acting via the bloodstream, which is consistent with them exerting paracrine rather than endocrine effects.[25-28] The signals from satiating peptides acting on the area postrema and on the vagus nerve are both received by the nucleus of the solitary tract (NTS) in the hindbrain, where they are integrated with other signals from the gastrointestinal tract, such as gastric distension.[29]

Ascending projections from the NTS go to numerous other brain regions, including the hypothalamus in the forebrain, which is an important control center of the autonomic nervous system.[30] One hypothalamic nucleus involved in energy homeostasis is the arcuate nucleus (ARC). In addition to receiving inputs from the NTS, the ARC is also the central site of action for the adiposity signals insulin and leptin. They exert their direct effects on food intake by stimulating pro-opiomelanocortin (POMC) neurons, which release the anorexigenic α-melanocyte stimulating hormone (αMSH), and by inhibiting neurons releasing the orexigenic transmitters agouti-related protein and neuropeptide Y (AgRP/NPY).[31] Other regions of the hypothalamus have also been shown to mediate various effects of leptin on energy homeostasis and glucose metabolism. The ventromedial nucleus of the hypothalamus seems to be involved in leptin-mediated regulation of blood glucose levels,[32] while the dorsomedial hypothalamus is necessary for the leptin-induced increase in sympathetic tone, which is thought to cause activation of brown adipose tissue thermogenesis.[33] The ventromedial hypothalamus[34] and the lateral hypothalamic area[35] are also critical for the control of body weight by leptin, which shows that a complex connectivity of different hypothalamic nuclei is required for leptin to exert its regulatory functions on energy homeostasis.

Furthermore, insulin and leptin also interact with gut peptide signaling in the hindbrain. By altering the sensitivity to short-term satiation signals, this provides an additional way to regulate long-term energy homeostasis.[36]

3.1.2. Energy expenditure

Although the hormonal regulation of food intake is quite well understood by now, that knowledge has so far not resulted in a successful weight loss drug that acts by decreasing long-term energy intake. This has recently led to an increasing interest in the other side of the energy balance equation, namely energy expenditure (EE). However, the determination of EE is technically much more challenging than the measurement of food intake and there are still many open questions to the analysis of EE data.

Total daily energy expenditure (TEE) of an animal consists of three main components. The major determinant is the basal metabolic rate (BMR), which contributes to about 60-75% of TEE.[37] BMR is the minimal energy that is required to maintain vital body functions and increases with increasing body mass; however, this correlation is not linear due to a higher influence of lean tissue mass compared to fat tissue mass.[38] Furthermore, lean tissue is metabolically not homogenous but comprises different organs, some of which are metabolically much more active (e.g. brain, kidneys, heart) than others (e.g. bone, skeletal muscle).[39,40]

The second component of TEE is diet-induced thermogenesis (DIT), also referred to as the thermic effect of food. It is defined as the increase in EE after a meal, which is associated with the digestion, absorption and storage of nutrients, and accounts for about 10-15% of TEE.[41] DIT does not only depend on the size of the ingested meal, i.e. its caloric content, but also on its nutrient composition. A protein-rich meal leads to a higher DIT compared to an isocaloric carbohydrate-rich meal, and a high fat content further decreases DIT.[37]

The third major contribution to TEE comes from activity related energy expenditure (AEE), which can be further divided into exercise and non-exercise activity thermogenesis. Non-exercise activity thermogenesis includes all muscle activity that not part of planned exercising, e.g. the energy expended for ambulation, posture or daily occupation.[42]

3.1.2.1. Control of energy expenditure

In contrast to the broad knowledge about food intake control, the processes and pathways regulating EE are still very poorly understood. This is to a large part due to the technical difficulties of EE measurement and the challenging data analysis (see below). In addition, the different components of EE seem to be controlled at least partly independent of each other, and their individual contribution to TEE is strongly influenced by environmental conditions. As mentioned previously, BMR is mainly determined by body mass and composition. However, it is strongly influenced by ambient temperature, since the maintenance of a constant body core temperature requires either active heat production at low ambient temperatures or physiological and behavioral responses to increase heat loss at high ambient temperatures, which both leads to an increase in BMR.[43] Assessment of BMR is relatively easy in rodents; it is usually done during the light phase when physical activity is low and after an overnight fast to exclude the effects of DIT.

AEE and DIT are more complex to evaluate since they are closely linked to each other under ad libitum fed conditions; i.e. an increase in EE caused by meal consumption is always associated with increased physical activity. Both can be calculated based on regression analyses by first evaluating the correlation between TEE and physical activity. If a good correlation is found, AEE can be estimated and subtracted from TEE; after this correction, measured changes in EE can theoretically be purely attributed to DIT. However, such analyses require a high sampling resolution and either a very high number of subjects or a long sampling interval.[44]

Interestingly, AEE is not only determined by the amount of physical activity but also by the energy cost of activity, which can vary widely between subjects and potentially provides one means of decreasing TEE in response to caloric restriction.[45] Even et al.[41] recently suggested that the energy cost of activity correlates with the efficiency of the coupling of O_2 consumption and ATP synthesis in muscles. This hypothesis is supported by studies showing a decreased expression of uncoupling-protein-3 in skeletal muscle in response to food restriction.[46,47]

Finally, DIT strongly depends on caloric content and composition of a meal. However, numerous other factors have been suggested to influence DIT, such as meal patterns,[48,49] exercise,[50,51] and insulin resistance associated with obesity.[52-54] Both exercise and obesity have been suggested to influence DIT by altering the activity of the sympathetic nervous system (SNS). SNS signaling to brown adipose tissue (BAT) is thought to be the main mechanism mediating DIT. How exactly this effect is mediated is not known, but there is evidence of a potential involvement of adiposity and satiation signals. Insulin and leptin act in the ARC to increase SNS activity.[55,56] Morbid obesity eventually leads to a reduction in insulin and leptin sensitivity, while exercise increases insulin and leptin sensitivity. Furthermore, some satiation signals have been shown to activate the SNS;[57,58] since insulin and leptin alter the sensitivity to satiation signals, insulin and leptin resistance could thereby further decrease SNS signaling indirectly.

3.1.2.2. Methods of energy expenditure measurement

As previously mentioned, the measurement of EE is technically challenging. The two options are direct calorimetry, which allows the accurate measurement of heat production in an animal, and indirect calorimetry, which estimates heat production based on an animal's oxygen consumption (V_{O2}) and carbon dioxide production (V_{CO2}). Direct calorimetry used to be the gold standard for EE measurement with the major advantage that the results do not depend on calculations based on generalized assumptions about metabolic functions.[59] However, the equipment is very expensive and complex.

Indirect calorimetry has become a popular and widely used alternative due to its commercial availability and comparably easy use. In contrast to direct calorimetry, the measurement of gas exchange also gives information about the substrates that are utilized for energy production. Due to the different C to O ratio in carbohydrate, lipid and protein, the amount of oxygen that is required for complete substrate oxidation is different among substrates, which leads to a specific V_{CO2}/V_{O2} ratio (respiratory quotient, RQ) for each substrate. The RQ expected for pure carbohydrate oxidation is 1.0, for pure fat oxidation 0.7. Protein oxidation or mixed substrates lead to RQ values between 0.7 and 1.0.[41] An RQ higher than 1 suggests a positive energy balance, i.e. the deposition of fat in response to carbohydrate ingestion, for example after long periods of fasting. One potential disadvantage of indirect calorimetry is that the calculation of EE from O_2 consumption and CO_2 production assumes a normally functioning metabolism. For example, it is assumed that there is

only negligible substrate interconversion, which is not the case during rapid changes in body weight, or that anaerobic metabolism only plays a negligible role.[59] It is therefore crucial that scientists using indirect calorimetry are aware of these limitations, especially since this method is often used to investigate genetically modified animals or effects of pharmacological and dietary interventions associated with various metabolic dysfunctions.

An additional problem regarding EE data analysis is the normalization of EE, more specifically the need to consider differences in body weight and body composition when comparing groups. The importance of this issue has become evident in the past few years with the increasing interest in genetically modified animals that display extreme abnormalities in both body weight and body composition. As mentioned before, BMR and therefore also TEE increase with increasing body mass, which led to the approach of normalizing EE for body weight. However, the correlation between BMR and body weight is not linear since weight gain in adults is usually associated with alterations in body composition, i.e. a higher percentage of fat mass. Normalizing EE for body weight therefore underestimates EE in obese animals, which has led to many controversial conclusions about the cause and effect of obesity-associated changes in EE. A frequently used alternative is the normalization for lean body mass, but this approach induces a very similar bias by overestimating EE in obese animals, because the additional energy produced by increased fat mass is attributed to the lean body mass. One option that has been proposed is the normalization for lean body mass + 0.2* fat mass.[41] The factor 0.2 is an estimation of the metabolic activity of adipose tissue compared to lean body mass; this formula should therefore provide a better approximation than the traditionally used ones. However, increased body mass not only affects BMR, but also AEE due to increased energetic cost of movement and probably DIT due to higher food intake and protein turnover.[60] The quantitative consideration of these components when analyzing differences in EE between animal groups with different body weights is extremely difficult, which is reflected in the number of recent publications on this issue in highly ranked journals.[41,44,61-64] It is recommended that, whenever possible, EE recordings should be performed before significant differences in body weight or composition between groups. This ensures that the causative differences, rather than consequences of the observed phenotypes, are measured.

3.1.2.3. Thermoneutrality

In addition to the question of how to compare EE between animals with differences in body weight and composition, the influence of the ambient temperature when performing metabolic studies is currently widely discussed. This issue first attracted broad attention due to mice lacking uncoupling protein-1 (UCP-1), which were expected to become spontaneously obese due to the incapability of DIT. Several studies performed at 20-23°C failed to detect such an effect[65-67] until Feldmann et al.[68] reported that UCP-1 ablation indeed induced obesity if the experiments were performed at thermoneutrality, i.e at 29°C. It was concluded that at lower temperatures, the large increase in EE

to maintain body core temperature masked potential differences due to the inability of BAT thermogenesis. Even though cold-induced thermogenesis is also mediated by UCP-1, there are other mechanisms that can compensate for a lack in cold-induced BAT thermogenesis, such as increased activity or shivering. However, at thermoneutrality the effects of UCP-1 ablation become evident since the UCP-1 dependent DIT accounts for an important fraction of TEE.

These findings clearly revealed the importance of considering housing temperatures when performing metabolic studies in rodents and have led to a rapidly growing body of literature on this issue. In order to maximize the utility of rodent models for the investigation of human diseases, particularly of those associated with metabolic abnormalities, it is now strongly recommended to perform experiments at thermoneutral temperatures.[69-71] The thermoneutral zone (TNZ) of an animal is defined by the temperature range within which metabolic rate is minimal and stable. The metabolic rate increases due to active heat production if ambient temperature falls below the lower critical temperature (LCT). If ambient temperature is elevated above the upper critical temperature (UCT), physiological and behavioral responses such as escape behavior, thermoregulatory grooming and increased breathing rate lead to a higher metabolic rate.[43] The LCT is relatively easy to determine by gradually lowering ambient temperature since the activation of heat-producing mechanisms directly correlates with an increase in metabolic rate. Reported values for the LCT of mice and rats vary, but seems to be around 30°C and 28°C for mice and rats, respectively.[72-76]

Determination of the UCT is more difficult because of different behavioral thermoregulatory responses that influence metabolic rate not as directly as adaptive thermogenesis. Changes in activity, water intake, evaporative water loss and skin temperature or skin blood flow should be included when defining the UCT. However, except for the distinct study of heat exposure effects, the UCT is of less practical interest than the LCT. It has been shown that some thermoregulatory responses, such as evaporative water loss and increased thermal conductance, are already activated when the LCT is reached and increase when ambient temperature is further elevated.[73] Therefore, experiments should ideally be performed at ambient temperatures only slightly above the LCT to minimize the influence of any thermoregulatory effects.

3.2. Dysregulation of energy homeostasis: The obesity epidemic

As discussed before, the hormonal control of food intake has been studied extensively. However, the results of experiments investigating the control of eating behavior are often very inconsistent even when performed under similar conditions.[77] This suggests that although energy homeostasis is a tightly controlled system, it is highly susceptible to external influences. The progressive rise in worldwide obesity prevalence supports the existence of such dysregulating factors, which currently attract huge interest in obesity research. Some of them have already been well characterized, including psychosocial stress,[78,79] addiction susceptibility or alterations in the reward system[80-82] and

prenatal influences,[83-85] the latter potentially leading to epigenetic changes and thereby also affecting future generations.[83] However, this increasing awareness of environmental factors leading to obesity has so far not resulted in any successful treatment or prevention options.

3.2.1. Current situation

Overweight and obesity with their resulting comorbidities have become a major topic in global healthcare and disease prevention. Overweight and obesity are defined by the World Health Organization as a Body Mass Index (BMI) of 25 or higher and 30 or higher, respectively. The BMI is a widely accepted index for obesity classification in adults, defined as a person's weight in kilograms divided by the square of the height in meters (kg/m^2).[86]

According to the World Health Organization, worldwide obesity has more than doubled since 1980; over 10% of the total adult population was considered obese in 2008. In recent years, obesity prevalence has also increased in low- to middle-income countries. In 2010, nearly 80% of the approximately 43 million overweight children under five lived in developing countries, indicating that rising problems are to be expected in particular in countries that might not have the financial possibilities to combat obesity and its consequences.[87]

3.2.2. Treatment options

The continuing increase in the worldwide obesity prevalence indicates that effective treatment options have not yet been identified or are at least not widely and easily accessible. The available options can be classified as conservative weight loss strategies, consisting of lifestyle and dietary modifications, as pharmacological treatment or surgical intervention.

3.2.2.1. Conservative weight loss strategies

Classical weight loss programs aim at reducing energy intake by dietary manipulation while increasing EE by enhancing physical activity. This leads to an energy deficit and thereby to a reduction in body weight by up to 10%;[88] however, the majority of patients regain a considerable amount of the lost weight after completing a closely supervised treatment.[89] This is not only the consequence of poor adherence to long-term therapies including profound lifestyle changes, but very likely also due to compensatory metabolic responses to the reduced body weight, such as a decrease in resting EE.[90] Overall, the metabolic changes after weight loss seem to favor weight regain.

3.2.2.2. Pharmacological treatment

Pharmacological treatment is considered in patients who fail to lose the intended amount of body weight by lifestyle changes alone, but there are many limitations to the use of pharmacotherapy in obesity treatment. The weight loss achieved with long-term drug therapy is generally smaller than

with strict adherence to diet modifications and activity increase,[91] and weight regain occurs as soon as the drug is discontinued.[92] In addition, safety concerns have repeatedly led to the withdrawal of popular weight loss drugs from the US and European market.[93,94] Although some new pharmacotherapies have recently been approved and many others are in the phase of clinical trials, their long-term effectiveness in morbidly obese patients have yet to be proven.[95,96]

3.2.2.3. Bariatric surgery

Surgical intervention is the treatment option for obese patients who have not responded to weight loss strategies or pharmacotherapy. It is by far the most effective option today and weight loss maintenance is achieved in many cases; further, it is the only treatment that leads to an improvement of the co-morbidities of obesity such as type 2 diabetes and cardiovascular disease. However, due to the associated risks of bariatric surgery, including micronutrient deficiency and mortality,[97] there are strict guidelines for the patients who qualify for surgery. In Switzerland, patients with a BMI of ≥35 (equates e.g. to a body weight of 113.5 kg for a person 1.80 m tall) are considered eligible for surgical intervention.[98]

Bariatric surgeries have traditionally been classified as malabsorptive, restrictive or combined procedures by the component that was thought to be mainly responsible for weight loss. Accordingly, gastric banding was considered to be a restrictive, jejunoileal bypass a malabsorptive and RYGB a mixed procedure.[99,100] However, the exact mechanisms behind most bariatric procedures are still poorly understood, and recent data indicate that endocrine mechanisms may not only underlie reduced eating after RYGB, but also after gastric banding.[101] Further, the rapid improvement of certain comorbidities that is observed after surgery seems to be independent of weight loss, pointing to more complex events.[102,103] Until recently, RYGB and adjustable gastric banding were the bariatric procedures that were most performed worldwide.[104] However, a relatively new procedure, vertical sleeve gastrectomy, has become very popular among bariatric surgeons in the past few years. It consists of resection of the greater curvature and fundus of the stomach, thereby strongly reducing gastric volume; it does however not involve any rearrangement of the intestinal anatomy. Although more invasive than the adjustable gastric band, it has now widely replaced this procedure because of its higher effectiveness.[105] The first short-term evaluations suggest that sleeve gastrectomy leads to similar weight loss as RYGB and there is also evidence that it may have beneficial effects on type 2 diabetes and cardiovascular disease.[100,106-108] However, in contrast to RYGB and adjustable gastric banding,[109] there is a lack of data regarding long-term sustainability of weight loss and diabetes remission after vertical sleeve gastrectomy.

3.3. Roux-en-Y gastric bypass

In spite of evolving less invasive new techniques with good short-term effects on weight loss, the RYGB is still considered the gold standard of bariatric procedures. It involves the creation of a small

gastric pouch, which is separated from the rest of the stomach and represents the restrictive element of the surgery. The duodenum and proximal jejunum remain attached to the stomach but are divided from the distal small intestine, thereby creating the “biliopancreatic limb”. The gastric pouch is then anastomosed to the distal remnant of the jejunum, creating the “alimentary limb”, and the biliopancreatic limb is reanastomosed distal to the gastrojejunostomy. This leads to the typical “Y” shape, in which food bypasses the major part of the stomach and the proximal small intestine. Food is mixed with bile and pancreatic enzymes only where the alimentary and the biliopancreatic limb are connected and form the “common channel”, the length of which was thought to determine the magnitude of malabsorption (Figure 1).

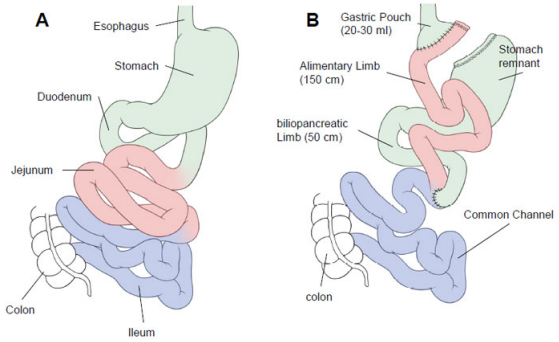

Figure 1 Illustration of the Roux-en-Y gastric bypass procedure. (A) Normal anatomy of the gastrointestinal tract, (B) gastrointestinal tract after gastric bypass surgery.

Intensive research on the weight loss mechanisms after RYGB surgery during the past years has led to many unexpected findings. Due to fast adaptations of the gastrointestinal tract to the anatomical rearrangement, the malabsorption observed immediately after surgery resolves quickly.[110,111] Therefore, nutrient malabsorption only plays a minor role for the resulting body weight loss, while alterations in caloric intake account for the larger portion of weight loss.[112] These changes in eating behavior are probably at least partly caused by differences in the postprandial release of satiating gut hormones, such as GLP-1, PYY and amylin.[113-115] It has been shown in rats that the effects of RYGB on food intake and body weight are partially abolished if the vagal nerve is not preserved during the surgery,[116] which supports an important role of gastrointestinal peptides in RYGB-induced weight loss, many of which act via the vagus nerve (see 3.1.1).

There are several other factors that might contribute to weight loss after RYGB. Studies in rat models of RYGB have shown a reduced preference for high fat diet[117,118] and alterations in sweet taste function,[119-121] and patients have repeatedly reported a shift in food preferences away from very sweet or fatty foods.[117] Miras et al. recently confirmed that the reward values of sweet and fat tastants were decreased in patients after RYGB compared to their preoperative values and to control subjects.[122] Further, changes in meal patterns towards smaller, but more frequent meals have been described and are associated with a reduced total food intake.[118] Recently, it has also been suggested that alterations in gut microbiota due to the anatomical changes of the digestive tract play a crucial role in RYGB-induced weight loss.[123] Finally, the effects of weight loss on EE are different after RYGB compared to caloric restriction, which could explain the better body weight maintenance after bariatric surgery.[124,125]

3.3.1. Effects of RYGB on energy expenditure

We and others reported that body weight loss in rats after RYGB is not associated with the same decrease in EE that is observed with traditional weight loss strategies.[118,124,125] Compensatory metabolic responses, such as decreased resting metabolic rate, are the main reason why maintenance of a lower body weight only by caloric restriction fails in the majority of obese patients[90]. The attenuation of this compensatory decrease in EE and in RYGB compared to control rats is therefore a very interesting and promising finding that requires further investigation in order to detect the underlying mechanisms.

There are several potential causes for the observed alterations in 24-hours EE. As mentioned above, the RYGB procedure leads to increased basal and postprandial release of GLP-1 from the distal small intestine. GLP-1 is the hormone that has most consistently been reported to be elevated after RYGB in humans and rats and is thought to be at least partly responsible for the reduction in food intake and the early improvement of glucose tolerance after surgery. Its effects on food intake have been characterized in numerous studies,[7,24,126,127] but GLP-1's role in the control of EE is less well investigated.

There are, however, a few studies that point to an involvement of GLP-1 in EE. Osaka et al.[57] showed a dose-dependent increase in oxygen consumption after intravenous GLP-1 administration, which was mediated by the lower brainstem and required the integrity of the sympathoadrenal system. Since GLP-1 levels rise rapidly in response to a meal and then return to baseline levels within one to two hours,[128] these data suggest that GLP-1 may be involved in mediating DIT. Even though other studies suggest a decrease of DIT by GLP-1 due to delayed gastric emptying,[129,130] Osaka's findings are interesting particularly in the context of the RYGB procedure. Due to the rearrangement of the gastrointestinal tract and the exclusion of the stomach as a temporary food storage organ, the confounding effects of GLP-1 on gastric emptying are less relevant after surgery; this then could explain differences in the role of GLP-1 in DIT between RYGB rats and rats with an intact stomach. Further evidence for a role of GLP-1 in EE control comes from mice lacking the GLP-1 degrading enzyme, dipeptidyl peptidase IV (DPP IV). These mice have more bioactive GLP-1 and are resistant to high fat diet-induced obesity because of reduced food intake and increased EE.[131] However, the results have to be interpreted with caution regarding their significance for GLP-1, since DPP IV not only degrades GLP-1, but processes many other peptides including PYY, oxyntomodulin (OXM) and GLP-2. Like GLP-1, postprandial levels of these hormones are increased after RYGB surgery,[113-115,132-134] and there are at least some studies showing an involvement of PYY[11] and OXM[135,136] in the control of EE.

GLP-2 has well-characterized, positive effects on epithelial proliferation particularly in the small intestine.[137] The resorptive surface is increased by enhanced crypt cell proliferation and reduced apoptosis of enterocytes.[138,139] It was therefore hypothesized that increased GLP-2 levels after

RYGB lead to mucosa hypertrophy and thereby limit malabsorptive consequences of the RYGB surgery, which in fact has been shown by Le Roux et al.[124,134] The gastrointestinal tract is a metabolically very active organ[140] and its hypertrophy is likely to contribute to the increase in EE after RYGB especially in response to a meal.

Therefore, it has to be considered that, similar to changes in food intake, the changes in EE in RYGB rats might be caused by the combined effects of several different factors rather than by just one specific gut hormone.

3.3.1.1. Brown adipose tissue and diet-induced thermogenesis

The elevation of the postprandial gut hormone response after RYGB has been reported repeatedly in rats and humans,[141-146] whereas the results on basal hormone levels are less consistent.[113,134,147-149] Therefore, if the alterations in EE that we observed in RYGB rats were at least partly caused by increased gut hormones, alterations in DIT might be expected.

As previously mentioned (see 3.1.2.1), DIT is mediated in part by the BAT.[150,151] In contrast to white adipose tissue (WAT), which is the primary organ for energy storage, the main function of BAT is the production of heat. Brown adipocytes are characterized by a large number of mitochondria that contain UCP-1. UCP-1 can uncouple mitochondrial respiration from ATP synthesis and thereby accounts for the thermogenic potential of BAT.[152,153] In addition, it is an excellent marker for BAT since its presence is unique to brown adipocytes.

The recent finding that BAT presence in adult humans correlated negatively with BMI, fasting glucose levels[154] and non-alcoholic fatty liver disease[155] led to increasing interest in the mechanisms behind BAT presence and activity.[154,156-159] This was further encouraged by the finding of emerging brown adipocytes in WAT depots. These adipocytes seem to transdifferentiate from mature white adipocytes or to differentiate from white preadipocytes,[160,161] distinguishing them from the ones found in classical BAT depots derived from myoblastic stem cells.[162,163] Differentiation of WAT into BAT has been shown in response to cold-exposure,[164] stimulation of β3-adrenergic stimulation,[165,166] and peroxisome proliferator-activated gamma (PPARγ) activation.[167] WAT to BAT differentiation also occurred spontaneously in a diet-induced obesity resistant rat model, possibly due to increased sympathetic innervation of WAT depots.[168,169] Together with the evidence suggesting an involvement of GLP-1 in the regulation of the sympathoadrenal system,[57] these findings may provide a connection between RYGB surgery, elevated GLP-1 levels and DIT.

3.3.2. Effects of RYGB on bone metabolism

3.3.2.1. Obesity-related changes in bone metabolism

Due to a positive correlation between BMI and bone mineral density (BMD),[170-173] the higher mechanical loading by increased fat mass is usually thought to be protective of bone mass. However, the interaction between fat and bone mass is far more complex than the traditional purely

weight-centered concept suggests and is influenced by diverse factors. Thus, even if the BMI/BMD correlation might be true in many cases, amounting evidence suggests that in individual subjects, this protective effect might be overridden by other obesity-associated mechanisms.[174-178]

It is well established that vitamin D deficiency occurs in obese people[179-181] and is mainly caused by increased vitamin D scavenging in fat compartments[180] and insufficient UV light exposure.[181] In accordance with this, presurgical evaluations of patients undergoing RYGB revealed low vitamin D levels in many cases, which was often accompanied by secondary hyperparathyroidism.[182,183] This compensatory response activates different mechanisms to prevent hypocalcemia, including increased calcium resorption from the bone reservoir.[184] Obesity can therefore affect bone metabolism by indirectly increasing parathyroid hormone (PTH) levels.

However, there are several additional factors that link adipose tissue and bone mass control. First, obesity leads to a chronic low-grade inflammatory state that is strongly involved in the development of obesity-associated comorbidities.[185] Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and C-reactive protein, are elevated in the adipose tissue and plasma of obese individuals.[186-189] Some of these cytokines, including TNF-α and IL-6, have been shown to stimulate osteoclast activity and bone resorption; a mechanism that also plays a role in postmenopausal osteoporosis.[190-192] Thus, obesity leads to changes in bone mass and quality by causing a chronic inflammatory response.

Second, plasma levels of the WAT-derived hormone leptin increase proportionally to total body fat mass.[193] Leptin potentially affects bone mass by contributing to the increase in proinflammatory markers.[194,195] Further, leptin has also been associated with bone mass control by affecting hypothalamic serotonin signaling leading to increased sympathetic activity.[56,196,197] However, while some studies suggest an inhibition of bone formation by leptin,[198,199] these findings are controversial and reports about the effects of leptin on bone mass diverge widely. Some studies show positive effects in different animal models, including leptin-deficient ob/ob mice,[200-203] others suggest differences between peripheral and central leptin signaling,[204] and human studies find neither an influence of leptin administration on bone metabolism,[205] nor a body weight-independent correlation between leptin levels and bone mass.[206] Therefore, even if leptin interacted with different factors that are involved in bone mass regulation, its overall role is unclear and leptin may in fact be of minor importance.

Third, dietary calcium and fat content influence the absorption of each other. Several studies in the past years have linked high dietary calcium intake with lower body weight gain and higher body weight loss compared to diets with normal calcium content.[207-210] This effect may in part be caused by the formation of insoluble calcium fatty acid soaps that lead to decreased fat absorption.[208,211,212] Conversely, maintenance on high fat diet has been shown to decrease calcium absorption and affect bone morphology.[213-215] The suggested mechanism behind these findings is a high fat diet-

induced redox imbalance particularly in the duodenum that leads to oxidative damage and down-regulation of genes involved in calcium absorption.[215] Since obesity is in many cases associated with the intake of foods containing high amounts of fat, reduced calcium absorption might be a widespread problem in the obese population.

Finally, adipocytes and osteoblasts originate from the same mesenchymal stem cells; an increase in bone marrow adipogenesis, as seen in aging, is accompanied by a decrease in osteogenesis.[216-218] However, this interaction is independent of whole body adiposity; bone marrow fat seems to be regulated differently and is, in contrast to other fat depots, not immediately affected by caloric restriction.[219] Recent studies suggest that the fat infiltration of bone marrow is not simply a consequence of reduced bone density as traditionally assumed. Instead, altered expression of local growth and transcription factors leads to predominant differentiation of mesenchymal stem cells into adipocytes.[220,221] PPARγ has been identified as the main transcription factor directing stem cell differentiation into adipocytes and thereby decreasing osteoblast differentiation.[217,222] Additionally to its suppressive effect on bone formation, PPARγ might also stimulate bone resorption by activating osteoclasts.[223,224] This is a very important finding, because thiazolidinediones, widely used in the therapy of type 2 diabetes, which is a primary co-morbidity of obesity, activate PPARγ, and increasing evidence suggests that these substances may lead to severe side effects on bone quality.[225,226]

Together, these data show that the influence of increased body adiposity on bone metabolism is complex and involves diverse factors. The consequences of obesity on bone quality can therefore vary between individuals.

3.3.2.2. Changes in bone metabolism after RYGB surgery

In accordance with the generally impaired vitamin D status in the obese population, vitamin D levels have repeatedly been shown to be low in patients before undergoing gastric bypass surgery. As RYGB decreases fat depots and usually also leads to a less sedentary lifestyle with more sunlight exposure, it would be expected to normalize calcium and vitamin D metabolism. Other factors associated with obesity that impair bone quality also improve after RYGB. Oxidative stress and inflammatory markers decrease soon after surgery,[227-229] and food preferences are reported to shift towards diets with a smaller fat content,[117,118,230] which should contribute to better calcium availability.

In contrast to this, the studies that evaluated bone metabolism after RYGB in humans mostly showed reduced BMD[231,232] or bone mineral content[233] that was accompanied by increased markers of bone resorption.[233-236] Even more frequently, an exacerbation of vitamin D deficiency and secondary hyperparathyroidism, in postsurgical follow-ups have been reported.[237-239] Most authors explain these findings by impaired absorption of fat and fat-soluble vitamins, such as vitamin D, due to the delayed mixing with bile and pancreatic enzymes.[240-242] Some state that calcium

malabsorption is the direct consequence of the insufficient vitamin D supply,[243] while others show evidence of a selective calcium malabsorption independent of vitamin D status.[240,244] Some studies also report secondary hyperparathyroidism after RYGB that is independent of the vitamin D status.[235,245,246]

The inconsistency of these results suggests that there may be unknown factors influencing bone metabolism after gastric bypass surgery. Especially the correlation between vitamin D levels and bone density has to be interpreted with caution, since there is a considerable seasonal variation in vitamin D levels.[238,247]

4. Aims of the project

Aim 1: To characterize longitudinal changes in bone metabolism after RYGB surgery in rats.
Increasing evidence from human studies suggests that RYGB surgery may cause bone loss. This has been attributed to vitamin D and calcium malabsorption, potentially leading to secondary hyperparathyroidism and increased bone resorption. However, the exact mechanisms are still unknown, and some studies have also shown bone loss in RYGB patients independent of vitamin D status. We therefore assessed bone mineral density, calcium and phosphorus balance, vitamin D levels, acid-base status and markers of bone turnover at different time points for 14 weeks after surgery in RYGB and sham-operated rats. In order to distinguish between effects of the surgery and weight loss per se, a control group was included that was body weight-matched to RYGB rats by food restriction.

Aim 2: To determine the effects of acute peripheral GLP-1 agonism and antagonism on energy expenditure and food intake in RYGB and sham-operated rats.
The compensatory decrease in EE after weight loss is attenuated in RYGB rats. GLP-1 may be involved in EE control, and basal and postprandial GLP-1 levels are increased after RYGB surgery. We hypothesized that increased GLP-1 levels are involved in RYGB-induced alterations in EE. We investigated the effects of acute peripheral administration of the GLP-1 agonist exendin-4 and the GLP-1 antagonist exendin-9 on EE in RYGB, sham-operated ad libitum fed and sham operated body weight-matched rats. The effects of exendin-4 and exendin-9 on food intake were evaluated to confirm successful drug administration and to test if RYGB surgery increases the satiating potential of both an exogenous GLP-1 agonist and endogenous GLP-1.

Aim 3: To assess changes in body composition after RYGB surgery
It has repeatedly been speculated that the reported alterations in EE could be caused by differences in body composition, i.e. a decreased fat mass proportion in RYGB rats. We therefore evaluated whole body composition of RYGB, sham operated ad libitum fed and sham operated body weight-matched rats with a validated rodent computer tomography scanner.

Aim 4: To identify the thermoneutral zone of RYGB and sham operated rats
Since the impact of ambient temperature when performing EE recordings in rodents has become obvious in the past few years, we hypothesized that a shift in the TNZ of RYGB rats towards higher temperatures may be responsible for their changes in EE when measured at ambient temperatures below thermoneutrality. We wanted to identify the TNZ of RYGB, sham operated ad libitum fed and

sham operated body weight-matched rats by gradually decreasing ambient temperature from 32 to 22°C and determine the temperature at which EE was lowest for each group.

I was also involved in other projects, which, although not directly related to the main focus of my thesis, provided additional insights into mechanism underlying weight loss and other metabolic effects of RYGB surgery. These included the investigation of (1) the influence of estrogen replacement in ovariectomized rats on body weight loss and the satiating potential of gut hormones after RYGB surgery, and (2) changes in gut microbiota composition in RYGB rats.

5. Original Research Article: “Roux-en-Y gastric bypass surgery reduces bone mineral density and induces metabolic acidosis in rats”

This section contains an original research article that was first submitted for publication to the American Journal of Physiology – Regulatory, Integrative and Comparative Physiology in January 2013 and accepted for publication in revised form in September 2013.

My contribution to this manuscript includes the study design, data acquisition, data analysis, data interpretation and drafting and revising the manuscript.

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Roux-en-Y gastric bypass surgery reduces bone mineral density and induces metabolic acidosis in rats

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Abegg K, Gehring N, Wagner CA, Liesegang A, Schiesser M, Bueter M, Lutz TA. Roux-en-Y gastric bypass surgery reduces bone mineral density and induces metabolic acidosis in rats. *Am J Physiol Regul Integr Comp Physiol* 305: R999–R1009, 2013. First published September 11, 2013; doi:10.1152/ajpregu.00038.2013.—Roux-en-Y gastric bypass (RYGB) surgery leads to bone loss in humans, which may be caused by vitamin D and calcium malabsorption and subsequent secondary hyperparathyroidism. However, because these conditions occur frequently in obese people, it is unclear whether they are the primary causes of bone loss after RYGB. To determine the contribution of calcium and vitamin D malabsorption to bone loss in a rat RYGB model, adult male Wistar rats were randomized for RYGB surgery, sham-operation–ad libitum fed, or sham-operation–body weight-matched. Bone mineral density, calcium and phosphorus balance, acid-base status, and markers of bone turnover were assessed at different time points for 14 wk after surgery. Bone mineral density decreased for several weeks after RYGB. Intestinal calcium absorption was reduced early after surgery, but plasma calcium and parathyroid hormone levels were normal. 25-hydroxyvitamin D levels decreased, while levels of active 1,25-dihydroxyvitamin D increased after surgery. RYGB rats displayed metabolic acidosis due to increased plasma lactate levels and increased urinary calcium loss throughout the study. These results suggest that initial calcium malabsorption may play a key role in bone loss early after RYGB in rats, but other factors, including chronic metabolic acidosis, contribute to insufficient bone restoration after normalization of intestinal calcium absorption. Secondary hyperparathyroidism is not involved in postoperative bone loss. Upregulated vitamin D activation may compensate for any vitamin D malabsorption.

Roux-en-Y gastric bypass; vitamin D; metabolic acidosis; secondary hyperparathyroidism

BARIATRIC SURGERY IS CURRENTLY the only effective long-term treatment for morbid obesity and its comorbidities. Roux-en-Y gastric bypass (RYGB) is the most commonly performed procedure and can be considered the gold standard for weight loss surgery. Recently, however, there has been an increasing focus on potential negative side effects of weight loss surgery, in particular, its effects on changes in bone metabolism (46, 49). Several prospective studies have reported significant decreases in bone mineral density (BMD) of the lumbar spine, hip, and femoral neck in men and women during the first year after RYGB surgery (13, 19, 39). The scarce data on long-term effects of RYGB surgery on bone mass suggest that reduced BMD may persist beyond the first postsurgical year (26, 50).

The anatomical rearrangement of the gastrointestinal tract after RYGB surgery leads to malabsorption of several micronutrients, including vitamin D (1, 19), and vitamin D deficiency is a common finding in patients after RYGB surgery (6). This may reduce intestinal calcium absorption (41), cause secondary hyperparathyroidism (2° HPT), and ultimately cause bone loss (19). Therefore, it has been proposed that postsurgical vitamin D levels should be regularly monitored and supplemented when necessary (43). However, several studies have reported a decrease in BMD after RYGB surgery despite vitamin D supplementation, indicating that vitamin D deficiency may not be the only cause of RYGB-associated changes in bone metabolism (12, 13, 39, 44). In addition, obesity per se is generally associated with low vitamin D levels due to vitamin D sequestration in adipose tissue and insufficient sunlight exposure (16). Therefore, presurgical evaluation of vitamin D and parathyroid hormone (PTH) status in patients undergoing RYGB often reveal substantial vitamin D deficiency and 2° HPT (21, 44). This complicates the assessment of postsurgical RYGB-induced changes and the interpretation of their specific contribution to bone loss. Finally, most studies report only values for 25-hydroxyvitamin D [25(OH)D] and not for the biologically active form 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]. To investigate the functional consequences of vitamin D malabsorption after RYGB surgery, measurement of 1,25$(OH)_2$D is indispensable. Interestingly, while presurgical levels of 1,25$(OH)_2$D have been reported to be decreased in RYGB patients (21), some studies report unaltered (1, 2) or even increased (44) postsurgical 1,25$(OH)_2$D levels. Overall, these conflicting results suggest that the role of vitamin D malabsorption as a single cause for postsurgical bone loss after RYGB seems unlikely and that more in-depth studies are required.

The aim of this longitudinal study was to characterize the time course of dynamic changes in bone metabolism after RYGB surgery in rats under standardized conditions without dietary calcium or vitamin D supplementation. We hypothesized that factors other than vitamin D deficiency may contribute to increased bone resorption and dysregulation of calcium homeostasis after RYGB.

MATERIALS AND METHODS

Animals and housing. Thirty-two adult male Wistar rats weighing 450–500 g were allocated to either RYGB surgery (n = 15), sham

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operation with no dietary manipulation [$n = 9$, ad libitum fed sham rats (AL)], or sham operation with food restriction to match the postoperative body weight of RYGB rats [$n = 8$, body weight-matched shams (BWm)]. RYGB and AL rats were housed in groups of four and three animals per cage, respectively; BWm rats were housed individually to allow food restriction. Animals were maintained under an artificial 12:12-h light-dark cycle at room temperature (24°C) and had ad libitum access to normal chow (Provimi Kliba, Kaiseraugst, Switzerland) and tap water unless otherwise stated. All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland.

Surgery. RYGB surgery was performed as previously described (10). Briefly, the small bowel was transected ~20 cm distal to the pylorus of the stomach, creating a proximal and distal end of small bowel. The proximal end constituted the biliopancreatic limb and was anastomosed to the ileum ~40 cm from the cecum, creating the common channel. The stomach was transected ~5 mm below the gastroesophageal junction, creating a gastric pouch of a size of no more than 2–3% of original stomach size. The Roux-en-Y reconstruction was completed by connecting the distal end of the small bowel to the gastric pouch leading to the formation of the alimentary limb. For sham operations, an anterior gastrotomy and a jejunotomy with subsequent closures were performed.

Microcomputed tomography scans. Two, seven, and fourteen weeks after surgery, BMD of lumbar vertebrae 1 to 6 was measured by quantitative microcomputed tomography (CT) with a LaTheta LCT-100A scanner (Hitachi-Aloka Medical, Tokyo, Japan). Rats were anesthetized with 2–3% isoflurane in oxygen (0.5 liter per min) during the measurement. Continuous 2-mm slice images with a pixel size of 250 × 250 μm were utilized for calculation of BMD using LaTheta software (version 2.10).

Sample collection. Rats were allowed to recover from anesthesia and CT scans for 1 day before being single-housed in metabolic cages (Tecniplast, Buguggiate, Italy) for urine and feces collection. Food consumption, water consumption, and body weight were recorded daily. After a 3-day adaptation period, urine and feces were collected for 24 h. Urine samples were collected under mineral oil to prevent evaporative losses. Rats were then anesthetized by inhalation of isoflurane/air, and venous blood was collected from the sublingual vein. Heparinized blood was immediately analyzed for pH, blood gases, and electrolytes on an ABL 800 Flex blood gas analyzer (Radiometer, Copenhagen, Denmark). Serum was stored at −20°C until further analysis. Sixteen weeks after surgery, rats were killed by decapitation after overnight fasting. To ensure comparability of the intestinal gene expression between animals, samples of the duodenum, jejunum, and ileum were taken 10 cm distal to the pylorus, 20 cm proximal to the cecum, and 60 cm proximal to the cecum, respectively.

Kidney and intestinal tissue was immediately frozen with liquid nitrogen and stored at −80°C for analysis by quantitative PCR and Western blotting.

Blood, urine, and feces analysis. Serum total calcium and phosphorus were measured using the chemistry analyzer Cobas Integra 800 (Roche Diagnostic System, Basel, Switzerland). Serum-intact parathyroid hormone and osteocalcin levels and urine total deoxypyridinoline crosslinks were measured by two-site enzyme-linked immunosorbent assays (iPTH: Immutopics, San Clemente, CA; MicroVue osteocalcin and MicroVue total DPD: Quidel, San Diego, CA), according to the manufacturer's protocols. Serum 25(OH)D and $1,25(OH)_2D$ levels were measured by radioimmunoassays (Immunodiagnostic Systems, Baldon, UK).

Ammonium in urine was measured by the method of Berthelot (7). For pH measurements of feces, 1 g of fresh feces was extracted with 5 ml of distilled water and centrifuged (10600 *g*, 20 min). pH of the feces extracts and urine pH were determined with a Metrohm 744 pH meter. Calcium and phosphorus in urine and feces were measured with the chemistry analyzer Cobas Integra 800. Duplicates of feces samples were dried at 105°C for 3 h to constant weight and then reduced to ashes in a muffle oven for 16 h at 550°C. The ash was dissolved in 10 ml 8% HCl, and the supernatant was used for analysis after centrifugation (3000 *g*, 10 min). Urine was acidified to pH <2.0 with HCl before analysis. Relative intestinal calcium and phosphorus absorption were calculated as relative absorption = [intake (mg) − fecal loss (mg)]/intake (mg).

RNA isolation and cDNA synthesis. Total RNA from kidney and intestine was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). Snap-frozen tissue was homogenized in a pestle homogenizer with 1 ml of precooled RLT buffer (Qiagen) supplemented with β-mercaptoethanol at a final concentration of 1%. Subsequently, 200 μl of the homogenate were used for RNA preparation, which was carried out according to the manufacturer's protocol. DNase digestion was performed using the RNase-free DNAase set (Qiagen). Total RNA extractions were analyzed for quality, purity, and concentration using the NanoDrop ND-1000 spectrophotometer. RNA samples were diluted to a final concentration of 100 ng/μl, and cDNA was prepared using the TaqMan reverse transcriptase reagent kit (Applied Biosystems/Roche, Foster City, CA).

In brief, in a reaction volume of 40 μl, 300 ng of RNA was used as a template and mixed with the following final concentrations of RT buffer (1×), $MgCl_2$ (5.5 mM), random hexamers (2.5 μM), dNTP mix (500 μM each), RNase inhibitor (0.4 U/μl), multiscribe reverse transcriptase (1.25 U/μl), and RNase-free water. Reverse transcription was performed with thermocycling conditions set at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min on a thermocycler (Biometra, Göttingen, Germany).

Real-time RT-PCR analysis. Semiquantitative real-time qRT-PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers for all genes of interest were designed using Primer Express Software (v.2.0.; Applied Biosystems), and primers were tested by PCR with cDNA and always resulted in a single product of the expected size. Primers were chosen to result in amplicons no longer than 150 bp spanning intron-exon boundaries to exclude genomic DNA contamination (Table 1). Probes were labeled with the reporter dye FAM at the 5′ end and the quencher dye TAMRA at the 3′ end (Microsynth, Balgach, Switzerland). Real-time PCR reactions were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). Briefly, 3.5 μl cDNA, 1 μl of each

Table 1. *Primers used for semi-quantitative real-time PCR*

Gene[1]	Accession No.[2]	Forward Primer (5′ - 3′) Reverse Primer (3′ - 5′)
TRPV5	NM_053787	AGA GCA GCC AAG GAA AAT GA
		TAG CAG CAT CCA GGT GGT CA
TPRV6	NM_053686	GCT GCA GCA GAA GAG GAT CT
		AGG GCA GCT ATG TGA AGT GC
Calb1	NM_031984	CCA CCT GCA GTC ATC TCT GA
		GCT CCT GGA TCA AGT TCT GC
Cyp24a1	NM_201635	TTG AAA GCA TCT GCC TTG TG
		GGG GTC ACC ATC ATC TTC C
Cyp27b1		Applied Biosystems Rn00678310_g1
Slc9a3 (NHE3)	NM_012654	CAC CCA CCA CAC GTT GCA
		GTG AGC TCG TGC CGA CTG T
Slc4a4 (NBCE1)	NM_053424	GGT GTG GAT ACT CCG AAG CTA ATT
		CAA AGG GTG GGA CAA ACC AA
HPRT	NM_012583	GCT GAA GAT TTG GAA AAG GTG TTT A
		ACA CAG AGG GCC ACA ATG TGA

[1]*TRPV5/6,* transient receptor potential vanilloid 5/6; *Calb1,* calbindin D28k; *Cyp24a1,* 1,25-dihydroxyvitamin D 24-hydroxylase; *Cyp27b1,* 25-hydroxyvitamin D 1-alpha-hydroxylase; *Slc9a3,* solute carrier family 9 (sodium/hydrogen exchanger) member 3; *Slc4a4,* solute carrier family 4 (sodium/bicarbonate cotransporter) member 4; *HPRT,* hypoxanthine guanine phosphoribosyl transferase. [2]NCBI Entrez Nucleotide Database (https://www.ncbi.nlm.nih.gov/nucleotide).

primer (25 μM), 0.5 μl labeled probe (5 μM), 6.5 μl RNase free water, and 12.5 μl TaqMan Universal PCR Master Mix reached 25 μl of final reaction volume. Reaction conditions were the following: denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 60 s with auto ramp time. All reactions were run in duplicate. For analyzing the data, the threshold was set to 0.06, as this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. The expression of the gene of interest was calculated in relation to hypoxanthine guanine phosphoribosyl transferase (HPRT). Relative expression ratios were calculated as $R = 2^{(Ct(HPRT)-Ct(test\ gene)}$, where Ct represents the cycle number at the threshold 0.06 (35).

Western blot analysis. Snap-frozen kidneys were homogenized in ice-cold RIPA buffer containing 1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 mM EDTA. After measurement of the protein concentration using the method of Pierce (45), 50 μg of proteins were solubilized in loading buffer containing β-mercaptoethanol and separated on 10% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically to nitrocellulose membranes. After blocking with 5% BSA in PBS/0.1% Tween-20 for 60 min, blots were incubated with the primary antibodies: mouse monoclonal anti-VDR D6 (51 kDa; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100) (51) and mouse monoclonal horseradish peroxidase (HRP)-conjugated anti-GAPDH (40 kDa; Abcam, Cambridge, UK; 1:10,000) overnight at 4°C. Membranes were then incubated for 1 h at room temperature with secondary goat anti-mouse antibodies 1:5,000 linked to HRP (Santa Cruz Biotechnology). The protein signal was detected with the appropriate substrates from Bio-Rad using the LAS-3000 chemiluminescence detection system (FujiFilm, Tokyo, Japan). All images were analyzed using the software ImageJ (20) to calculate the VDR/GAPDH ratio.

Bone histology. Bone specimens were fixed in 40% ethanol at 4°C for 3 days and further dehydrated in an ascending series of ethanol (50%, 70%, 80%, 90% 96%, and 100% at 4°C, 2 days per concentration) before being defatted in xylene under vacuum for 4 days. Embedding of specimens in methylmetacrylate was performed in customized Teflon forms. Tibiae and L3 vertebral bodies were embedded in a position that allowed midsagittal and coronal sectioning, respectively. After polymerization, the blocks were mounted on plastic frames and cut with a precision saw (Leica SP 1600, Leica Biosystems, Nussloch, Germany). Ground sections were mounted on acrylic glass slides (Perspex GS Acrylglas Opal 1013; Wachendorf AG, Basel, Switzerland), polished to 200-μm sections, and surface stained with toluidine blue. Sections were digitally recorded (Axiovision, Carl Zeiss AG, Oberkochen, Germany; magnification 10 × 4); bone volume fraction and trabecular thickness were quantified with the software ImageJ and the plug-in BoneJ (15, 17) in vertebral bodies and proximal tibiae. To exclude the primary spongiosa from the analysis, cancellous bone within 0.5 mm from the growth plate and within 0.25 mm from the endocortical bone surface was excluded (18).

For the measurement of growth plate width, images of the total growth plate were obtained at a magnification of 10 × 10. Seven vertical (perpendicular to chondro-osseous junction) lines were drawn throughout the growth plate and measured; growth plate width was calculated as an average of these seven measurements (23).

Statistical analysis. Data were analyzed with one-way ANOVA and are expressed as means ± SE. Following a significant F ratio, Bonferroni-Hochberg post hoc tests were used to determine differences between groups. Significance was established at $P < 0.05$.

RESULTS

RYGB surgery leads to body weight loss and maintenance of a lower body weight. RYGB rats lost ~15% of their original body weight within the first 2 wk after surgery and maintained a significantly lower body weight compared with AL rats from postoperative *day 4* ($P < 0.05$). From *week 2* after surgery, body weight was significantly lower in BWm rats compared with AL rats ($P < 0.001$), but there was no difference between RYGB and BWm rats (Fig. 1). We have previously shown that BWm rats require significantly less food than RYGB rats to maintain a similar body weight and that the lower food efficiency in our RYGB model is not caused by nutrient malabsorption, but it is associated with an increase in energy expenditure (11).

BMD decreases after RYGB surgery. BMD was significantly reduced in RYGB rats already 2 wk after surgery and decreased further between *weeks 2* and *7*; thereafter, the difference in BMD between RYGB and AL rats remained unchanged until *week 14*. There was no difference in BMD between AL and BWm rats at any time point, which suggests that the decrease of BMD in RYGB rats was not directly caused by body weight loss (Fig. 2*A*). The results obtained by CT were confirmed by bone histomorphometry, which showed reduced bone volume and trabecular thickness in vertebrae and reduced bone volume in tibiae of RYGB, but not BWm rats (Fig. 2, *B–F*).

RYGB leads to transient calcium malabsorption and increased urinary calcium excretion. To determine the potential role of malabsorption in the observed bone changes, calcium and phosphorus absorption was calculated from 24-h intake and fecal losses. Two weeks after surgery, both the absorption relative to intake and the absolute absorption of calcium were lower in RYGB rats compared with sham controls. However, there was no evidence of calcium malabsorption in RYGB rats 7 and 14 wk after surgery (Fig. 3*A*). The absorption of phosphorus was not impaired at any time point in RYGB rats (Fig. 3*B*). Fourteen weeks after surgery, there was a decrease in calcium and phosphorus absorption compared with the earlier time points in all rats. We speculate that this was because rats had reached the age when linear growth stopped (Fig. 1), since it is known that mineral absorption is markedly decreased in older rats (3).

We expected RYGB rats to compensate for the initial calcium malabsorption by decreasing urinary calcium excretion. Paradoxically, urinary calcium and phosphorus concentrations, as well as the absolute 24-h excretions, were increased in RYGB rats throughout the entire study (Fig. 3, *C* and *D*).

RYGB causes chronic metabolic acidosis. Because overall bone metabolism and renal calcium excretion are strongly affected by acid-base imbalances, we investigated systemic acid-base status after RYGB surgery. Throughout the study,

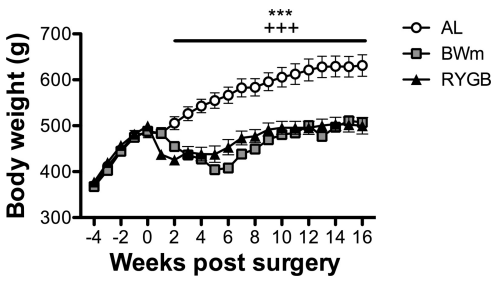

Fig. 1. Body weight changes of sham-operated, ad libitum-fed (AL), sham-operated, body weight-matched (BWm), and Roux-en-Y gastric bypass (RYGB) rats. Data are expressed as mean values ± SE (***$P < 0.001$ AL vs. RYGB; +++$P < 0.001$ AL vs. BWm).

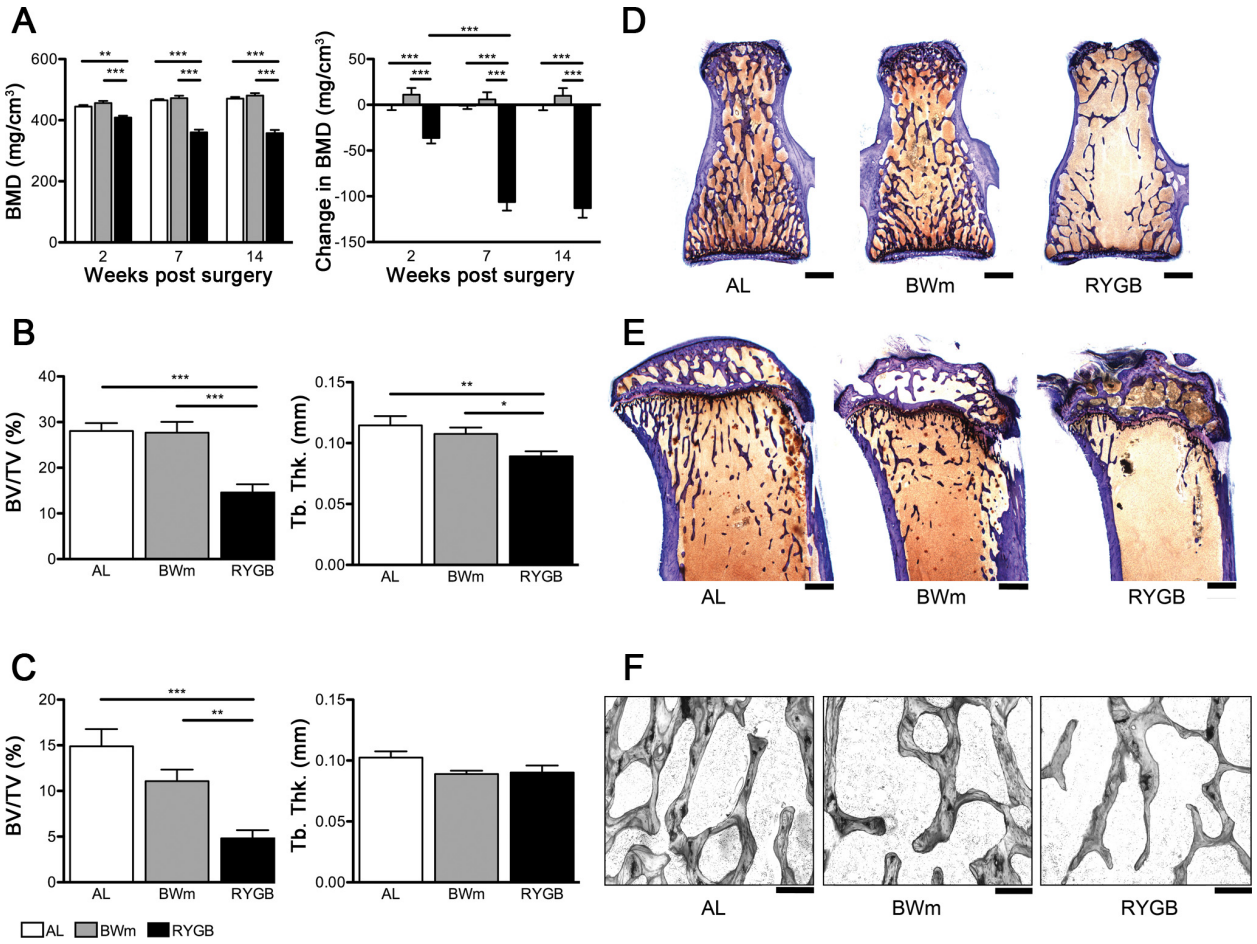

Fig. 2. Decreased bone mass in RYGB rats. *A*: bone mineral density (BMD) of lumbar vertebrae measured by computed tomography (CT) and change in BMD compared with AL rats 2, 7, and 14 wk after surgery. *B* and *C*: bone volume/trabecular volume fraction (BV/TV) and trabecular thickness (Tb. Thk.) of lumbar vertebrae (*B*) and tibiae (*C*) evaluated by histomorphometric analysis 16 wk after surgery. *D* and *E*: representative images of toluidine blue stained L3 vertebral bodies (*D*) and tibiae (*E*) of AL, BWm, and RYGB rats. *F*: higher magnification illustrating reduced trabecular thickness in L3 vertebral bodies of RYGB, compared with AL and BWm rats. Data represent mean values ± SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Bar = 1 mm (*D* and *E*); 200 μm (*F*).

venous blood pH was significantly decreased in RYGB compared with sham rats, and the anion gap and lactate levels were increased (Fig. 4*A*). There was a significant negative correlation between lactate levels and blood pH in RYGB rats ($R = -0.667$), but not in AL or BWm rats, suggesting that increased lactate levels at least partly accounted for the metabolic acidosis of RYGB rats.

Chronic metabolic acidosis (CMA) leads to an activation of compensatory mechanisms in the kidney and intestine, including increased renal ammonium excretion and upregulation of the intestinal ion transporters sodium-hydrogen exchanger 3 (NHE3) and sodium-bicarbonate cotransporter 1 (NBCe1) (31). Accordingly, urinary and fecal pH were decreased (Fig. 4*B*) in RYGB rats, while urinary ammonium excretion was increased (Fig. 4*C*). mRNA expression of NHE3 was increased in the jejunum and of NBCe1 in the jejunum and ileum of RYGB rats, respectively (Fig. 4*D*). Together, these results indicate an adaptive response to the CMA in RYGB rats; however, this response seemed to be insufficient to fully compensate for the increased lactate levels and systemic acidosis.

Changes in parameters of mineral homeostasis and bone metabolism after RYGB surgery. Total and ionized serum calcium levels were unchanged in RYGB rats except for a small decrease after 14 wk compared with AL rats. Serum phosphorus levels were increased compared with BWm, but not AL rats 2 wk after surgery. There was no difference 7 or 14 wk after surgery (Fig. 5*A*). Surprisingly, there was no significant difference between PTH levels of RYGB and sham-operated rats at any time point (Fig. 5*B*).

Serum osteocalcin levels and urinary deoxypyridinoline (DPD) excretion were measured as markers of bone formation and resorption, respectively. While osteocalcin was unchanged throughout the study, DPD excretion was increased compared with BWm rats at 2 and 7 wk after surgery, suggesting increased bone resorption at these time points, at least compared with weight-matched animals (Fig. 5*C*).

Renal activation of vitamin D is increased, but vitamin D-induced gene upregulation in the kidney is lower after RYGB. Consistent with human studies reporting 25(OH)D deficiency in patients after RYGB surgery, RYGB rats had significantly lower levels of 25(OH)D than both AL and

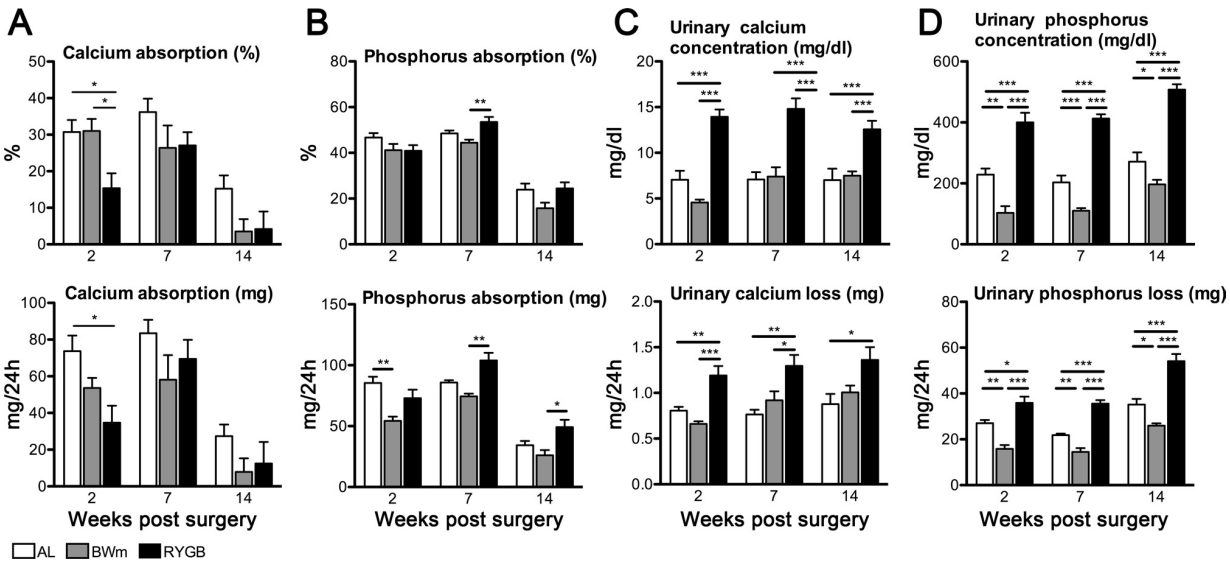

Fig. 3. Relative and absolute 24-h intestinal absorption of calcium (*A*) and phosphorus (*B*), 24-h urinary concentration and absolute urinary loss of calcium (*C*) and phosphorus (*D*) in AL, BWm, and RYGB rats 2, 7, and 14 wk after surgery. Data are expressed as means ± SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

BWm control rats. However, active $1,25(OH)_2D$ was increased more than twofold (Fig. 6*A*). Consistent with the circulating blood levels, RYGB rats displayed both increased renal expression of the vitamin D-activating enzyme 1-α-hydroxylase (CYP27B1) mRNA and decreased renal expression of the vitamin D-inactivating enzyme 24-hydroxylase (CYP24A1) mRNA (Fig. 6*B*), suggesting that both increased activation and decreased inactivation contributed to elevated circulating $1,25(OH)_2D$ levels.

The vitamin D receptor (VDR) is a nuclear transcription factor that is expressed in various tissues (51). Binding of $1,25(OH)_2D$ to the VDR leads to upregulation of genes involved in calcium homeostasis, including the VDR itself, to increase intestinal calcium absorption and decrease renal calcium excretion. In the kidney, the membrane calcium channel transient receptor potential vanilloid 5 (TRPV5) and the cytosolic calcium buffer calbindin D28k (CALB1) are upregulated by $1,25(OH)_2D$ (53); VDR binding in the intestine leads to upregulation of TRPV6 under normal conditions (28). Nevertheless, mRNA expression of TRPV5 and CALB1 in RYGB rats was unchanged (Fig. 6*C*), and VDR protein levels were lower in the kidney of RYGB rats (Fig. 6*D*). In contrast, $1,25(OH)_2D$ signaling in the intestine seemed to be intact because TRPV6 mRNA expression was increased in the duo-

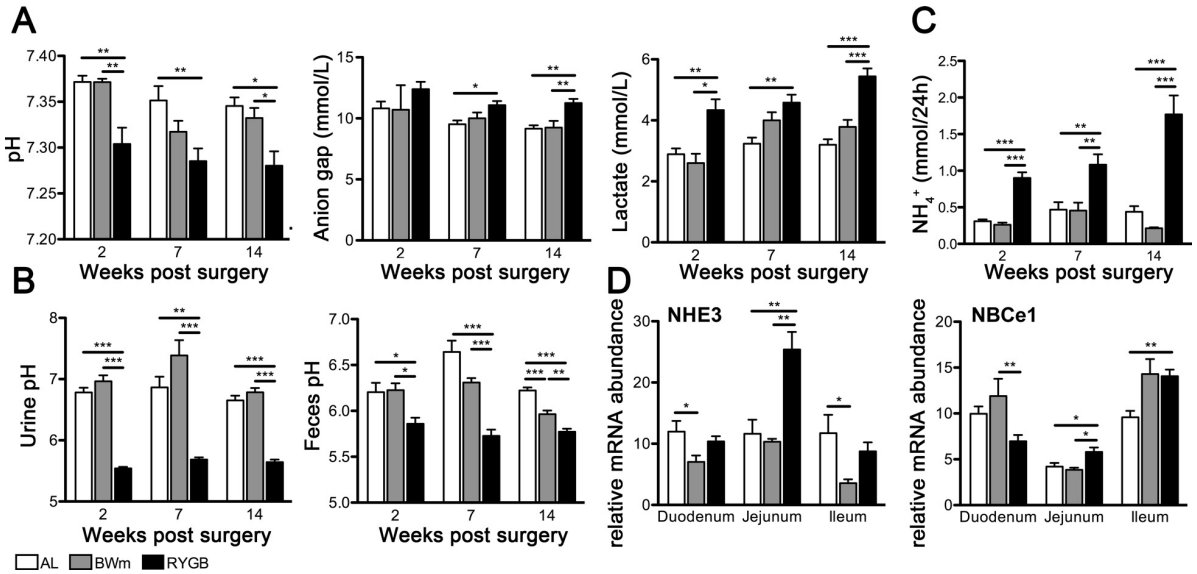

Fig. 4. Venous blood pH, anion gap, and lactate levels (*A*), pH of urine and feces extracts (*B*), and 24-h urinary ammonium (NH_4^+) excretion (*C*) in AL, BWm, and RYGB rats 2, 7, and 14 wk after surgery. *D*: mRNA expression of the apical sodium-hydrogen exchanger NHE3 and the basolateral sodium-bicarbonate cotransporter NBCe1 in duodenum (biliopancreatic limb in RYGB), jejunum (alimentary limb in RYGB), and ileum (common channel in RYGB) of AL, BWm, and RYGB rats 16 wk after surgery. Data are expressed as means ± SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

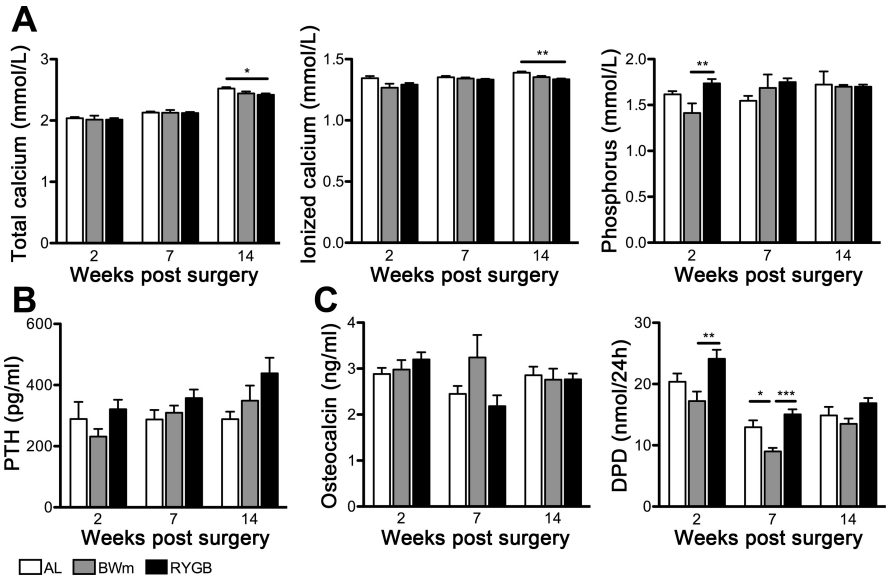

Fig. 5. Serum levels of total calcium, ionized calcium, and phosphorus (*A*) and of parathyroid hormone (PTH) (*B*). *C*: serum levels of osteocalcin and urinary 24-h deoxypyridinoline (DPD) excretion in AL, BWm, and RYGB rats 2, 7, and 14 wk after surgery. Data represent mean values ± SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

denum and jejunum of RYGB rats, as expected. TRPV6 expression was also upregulated in the duodenum of BWm rats, which may potentially be considered an adaptive response to reduced dietary calcium intake (Fig. 6*E*).

Increased 1,25(OH)$_2$D levels in RYGB rats potentially affect growth plate maturation. Vitamin D deficiency or lack of the VDR lead to characteristic changes in the epiphyseal growth plates, i.e., growth plate widening with disorganized structure and accumulation of immature, unmineralized bone mass (8). 1,25(OH)$_2$D on the other hand reduces growth plate width in young rats (23). In our study, the histological evaluation of the epiphyseal growth plate in the proximal tibia revealed a trend toward narrowing of the growth plates in adult RYGB rats ($P < 0.1$; Fig. 7).

DISCUSSION

We performed the first comprehensive longitudinal investigation of changes in bone metabolism after RYGB surgery in a rat model. Postoperative bone loss has repeatedly been described in RYGB patients and is often associated with low 25(OH)D levels and 2° HPT (6, 14, 25). To our knowledge, however, no causal relationship among vitamin D status, PTH levels, and bone loss after RYGB surgery has been demon-

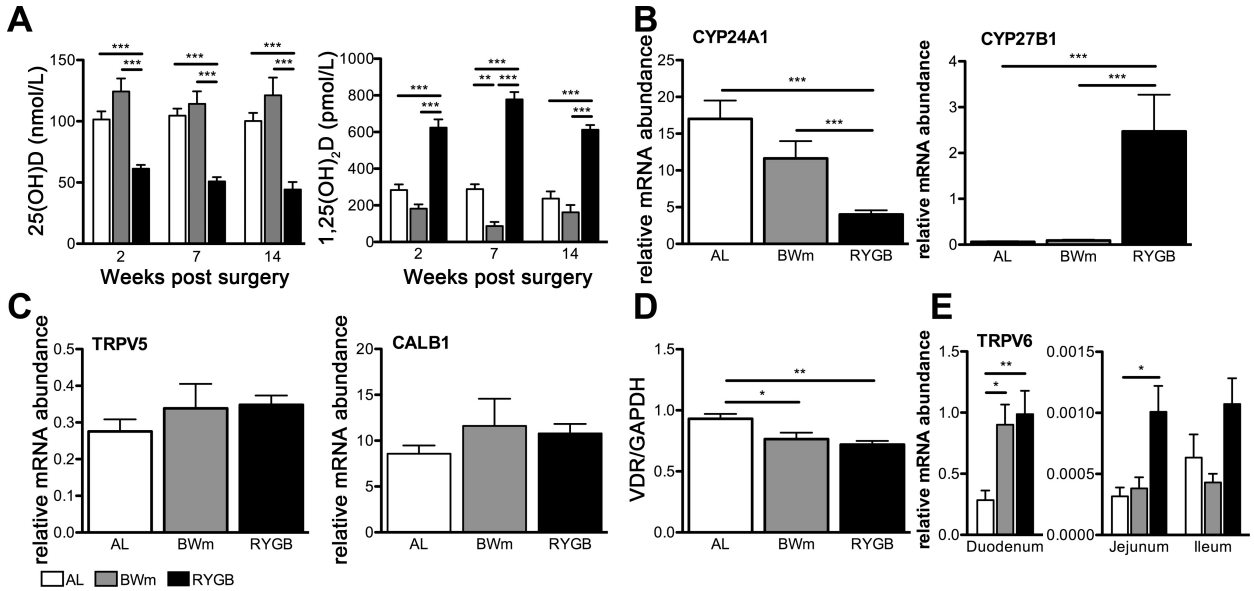

Fig. 6. Altered vitamin D metabolism and signaling after RYGB surgery. *A*: serum levels of 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] in AL, BWm, and RYGB rats 2, 7, and 14 wk after surgery. *B–D*: renal mRNA expression of CYP24A1 and CYP27B1, the genes encoding the 24-hydroxylase and the 1-α-hydroxylase, respectively (*B*), renal transient receptor potential vanilloid 5 (TRPV5), and calbindin D28k (CALB1) mRNA expression (*C*) and renal vitamin D receptor (VDR) protein expression (*D*) in AL, BWm, and RYGB rats 16 wk after surgery. *E*: TRPV6 mRNA expression in duodenum, jejunum, and ileum of AL, BWm, and RYGB rats 16 wk after surgery. Data represent mean values ± SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

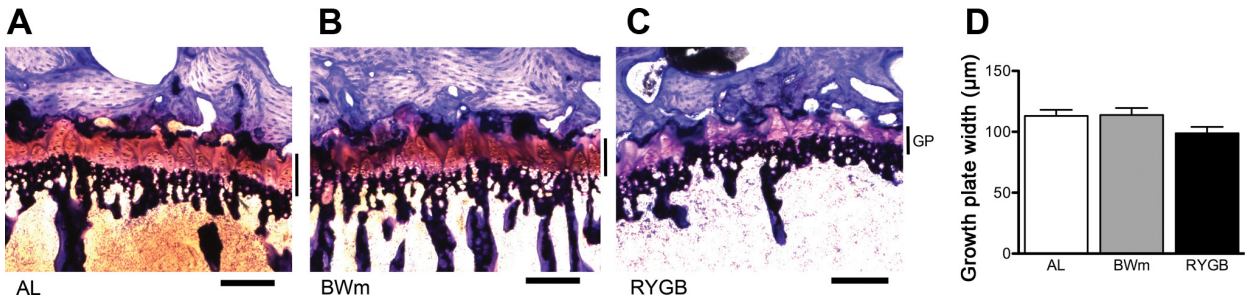

Fig. 7. Representative images showing growth plates of toluidine blue-stained tibiae of sham-operated, ad libitum fed (AL; *A*), sham-operated, body weight-matched (BWm; *B*) and Roux-en-Y gastric bypass-operated (RYGB; *C*) rats and quantification of growth plate width (*D*) 16 wk postsurgery. Data are expressed as mean values ± SE. Scale bar: 100 μm.

strated. Since vitamin D deficiency and 2° HPT often occur in obese patients even before any weight loss intervention, it is difficult to dissect the specific contribution of RYGB surgery to these conditions. Using a well-established RYGB rat model with standardized light exposure and access to standard laboratory chow without calcium or vitamin D supplementation, we showed that BMD decreased progressively for several weeks after surgery. Bone loss was initially associated with calcium malabsorption; however, no restoration of bone mass was detectable in RYGB rats even after normalization of intestinal calcium absorption. No signs of 2° HPT were detected, but elevated active vitamin D levels and metabolic acidosis caused by increased lactate levels occurred throughout, which may have adversely affected bone metabolism. These data suggest that initially, reduced calcium absorption after RYGB may lead to calcium mobilization from bone; however, this seems to be independent of vitamin D malabsorption. In contrast, RYGB-induced metabolic acidosis may interfere with the hormonal control of calcium homeostasis and bone mass. Importantly, these changes were independent of body weight loss, since they did not occur in the food-restricted, body weight-matched control group.

Our data show that after an initial decrease in BMD, the difference between AL and RYGB rats remained unchanged between postoperative *weeks 7* and *14*. Together with the normalization of intestinal calcium absorption at these time points, this suggests that impaired intestinal calcium absorption contributed to bone loss, at least initially. Under physiological conditions, most of dietary calcium is absorbed in the duodenum (42) by active transcellular transport, which requires the TRPV6 calcium channel (29). TRPV6 is almost exclusively expressed in the duodenum and is upregulated in response to increased $1,25(OH)_2D$ levels. In the distal small intestine, passive calcium absorption is predominant (38). The passive absorption strongly depends on the luminal calcium concentrations and is, therefore, lower at low dietary calcium intake. After RYGB surgery, the proximal small intestine is excluded from nutrient flow, most likely leading to impaired transcellular calcium absorption. However, the direct delivery of nutrients to more distal parts of the small intestine and the increased luminal calcium concentrations could lead to increased passive calcium absorption in the jejunum and ileum. A recent study by Pan et al. (37) suggests that the sodium bicarbonate exchanger NHE3 is involved in passive calcium absorption. Interestingly, NHE3 mRNA expression was increased in the jejunum of RYGB rats, which may enhance passive calcium absorption in the distal small intestine. Thus, the initial calcium malabsorption observed in RYGB rats is most likely caused by the absence of active transport in the excluded proximal small intestine. The upregulation of TRPV6 expression in the duodenum and jejunum of RYGB rats indicates a compensatory response to the sudden decrease in calcium availability. This response, however, is futile since the proximal small intestine is excluded from nutrient flow; nonetheless, the data show that the duodenum of RYGB rats remains responsive to elevated $1,25(OH)_2D$ levels, even in the absence of direct nutrient contact. The compensation by increased passive absorption in the distal small intestine seems to be insufficient shortly after surgery, potentially due to a lack of adaptation time of the gut to the altered anatomy. Duodenal TRPV6 expression was also increased in BWm rats; however, their $1,25(OH)_2D$ levels were comparable to AL rats. It has been described in animals on a calcium-deficient diet that reduced dietary calcium intake leads to increased $1,25(OH)_2D$-mediated TRPV6 expression (48, 52); it is, therefore, surprising that the food-restricted BWm rats did not display increased $1,25(OH)_2D$ levels. It must be noted, however, that in contrast to animals fed a calcium-deficient diet ad libitum, our animals were fed a restricted amount of a diet with normal calcium content. This might physiologically represent a completely different situation since, unlike a calcium-deficient diet, our approach presumably does not influence the luminal calcium concentration in the intestine. To our knowledge, the effects of chronic food restriction on $1,25(OH)_2D$ levels and on intestinal calcium transporter gene expression have never been investigated. Therefore, we speculate that in this situation, either a vitamin D-independent mechanism or a change in vitamin D receptor sensitivity led to duodenal TRPV6 upregulation.

The finding of increased PTH levels in many patients after RYGB surgery has led to the assumption that PTH-induced bone resorption is the main cause of reduced BMD. However, the high prevalence of 2° HPT in obese people already before RYGB surgery complicates the assessment of a potential causal link between PTH and postsurgical bone loss. Interestingly, we did not find a significant increase in PTH levels in RYGB rats, which suggests that PTH-independent mechanisms causing bone loss seem to prevail in the first weeks after surgery.

Consistent with previous studies reporting 25(OH)D deficiency in patients after RYGB surgery, our RYGB rats had decreased 25(OH)D levels. Importantly, however, we found that levels of the hormonally active metabolite $1,25(OH)_2D$ were markedly increased. Even though $1,25(OH)_2D$ increases

active calcium absorption under physiological conditions, we cannot exclude that in combination with intestinal calcium malabsorption, $1,25(OH)_2D$ may, in fact, have negative effects on bone mass. A recent study by Lieben et al. (30) showed that specific disruption of intestinal vitamin D signaling in intestine-specific VDR knockout mice increased $1,25(OH)_2D$ levels, which contributed to maintaining normocalcemia by mobilization of skeletal calcium and inhibition of bone mineralization. Because the main physiological effect of intestinal vitamin D signaling is to increase active calcium absorption in the proximal intestine, intestine-specific VDR knockout mice may, to some extent, be comparable to the situation after RYGB surgery, where duodenal vitamin D signaling, although intact, remains ineffective, due to the altered gut anatomy. We, therefore, speculate that increased $1,25(OH)_2D$ levels contribute to bone loss in the initial phase of intestinal calcium malabsorption and to the insufficient restoration of bone mass after normalization of calcium absorption (22).

The exact mechanism behind increased $1,25(OH)_2D$ levels in our RYGB rats is not clear. The fact that $1,25(OH)_2D$ levels remained elevated after normalization of calcium absorption suggest that the increased vitamin D activation may not only be related to serum calcium levels. Our data show that RYGB surgery led to severe systemic CMA that was at least partly due to increased plasma lactate levels. We did not determine the source of increased lactate levels; however, we speculate that alterations in the bacterial flora of the intestine (54) led to increased lactate production after carbohydrate digestion, a phenomenon frequently observed in patients with short-bowel syndrome or jejuno-ileal bypass surgery (40). CMA leads to a PTH-independent increase in $1,25(OH)_2D$ production (27) and could, therefore, play a role in vitamin D metabolism after RYGB surgery. In addition, it has previously been speculated that enterocytes may have the capacity to sense changes in dietary calcium intake and to increase renal 1-α hydroxylase activity via an unknown signal in response to decreased intestinal calcium concentrations (32). The sudden absence of luminal calcium in the proximal small intestine after the RYGB procedure could, thereby, directly induce an increase in $1,25(OH)_2D$ levels.

An unexpected finding of our study was that renal calcium excretion was increased in RYGB rats; this was already seen in the initial phase of the study with intestinal calcium malabsorption and massive bone loss, but also later in the study. A decrease in intestinal calcium availability normally leads to $1,25(OH)_2D$-mediated upregulation of renal CALB1 and TRPV5 expression (53) to minimize urinary calcium loss. Despite higher $1,25(OH)_2D$ levels, urinary calcium concentration and absolute calcium excretion were increased in RYGB rats throughout the study, and there was no change in renal CALB1 and TRPV5 expression when examined at study end. This suggests that additional factors may have counteracted the control of renal calcium metabolism by $1,25(OH)_2D$. CMA has been shown to enhance calciuresis by decreasing the expression of CALB1 and TRPV5 in the kidney (34); therefore, the CMA detected in RYGB rats is one possible explanation for increased urinary calcium loss. CMA has additional direct effects on bone metabolism, including a stimulatory effect of H^+ on osteoclast activation (4) and an inhibition of bone formation and mineralization (9). CMA may, therefore, play an important facilitator role in bone resorption after RYGB surgery and in the prevention of bone mass restoration after normalization of calcium absorption.

Since the original submission of our paper, Stemmer et al. (47) published data on the effect of RYGB surgery on bone mass in rats. Our data are largely consistent with their findings. Similar to our results, Stemmer et al. (47) found that bone mass was reduced by RYGB 8 wk after surgery, which was associated with decreased 25(OH)D levels. A calcium and vitamin D-enriched diet attenuated the effects of RYGB surgery on bone mass; however, it did not fully prevent bone loss, which further suggests that additional factors to calcium and vitamin D malabsorption may be involved in RYGB-induced bone loss. In addition to their findings, we now report that lower 25(OH)D may not be a pathophysiologically relevant factor because increased conversion to the active metabolite may compensate for lower absorption. Hence, our data nicely complement the findings by Stemmer et al. (47) and suggest further mechanisms that may contribute to the significant bone loss that occurs after RYGB in rats.

Relevance to human disease. The current study provides valuable new insights into the effects of RYGB surgery on bone metabolism that may be of high clinical interest (Fig. 8). The increased levels of active $1,25(OH)_2D$ in our RYGB rats indicate that vitamin D deficiency may not be the sole cause of RYGB-induced bone loss (44) because insufficient 25(OH)D absorption may be compensated by a higher proportion of transformation to the active $1,25(OH)_2D$ form of vitamin D. This finding suggests that the need of vitamin D supplementation after surgery should be evaluated on the basis of measured 25(OH)D and $1,25(OH)_2D$ levels. Further, we believe that calcium supplementation may have beneficial effects on bone mass, especially in the first months after the procedure, because calcium malabsorption was transient and because there was no further bone loss in our RYGB rats after normalization of calcium absorption. It has to be mentioned in this context that the jejunojejunostomy in our rat model is performed more distally than in most human RYGB procedures. This could potentially contribute to the reported calcium malabsorption; however, it has previously been shown that the true fractional calcium absorption also decreases after RYGB surgery in humans (41), suggesting that this observation is not specific to our model but of general relevance. Finally, metabolic acidosis should be monitored and treated with alkaline supplementations, if necessary. Alkaline treatment has been shown to increase bone mass, even in nonacidotic postmenopausal women (24).

Even though the clinical relevance of bone loss after RYGB surgery in humans is still unclear, there are at least two patient subpopulations that are of particular interest for potential future studies. First, a majority of patients undergoing RYGB surgery are women, and many of them are perimenopausal or postmenopausal. We have recently established an RYGB model in female rats and have shown that the reproductive axis function may influence the outcome of RYGB surgery in women (5). Given the strong connection between menopause and osteoporosis, it will be of high interest to determine whether the menopausal state directly influences bone loss after RYGB surgery and whether there are potential interactions between the mechanisms of postsurgical and postmenopausal bone loss in women.

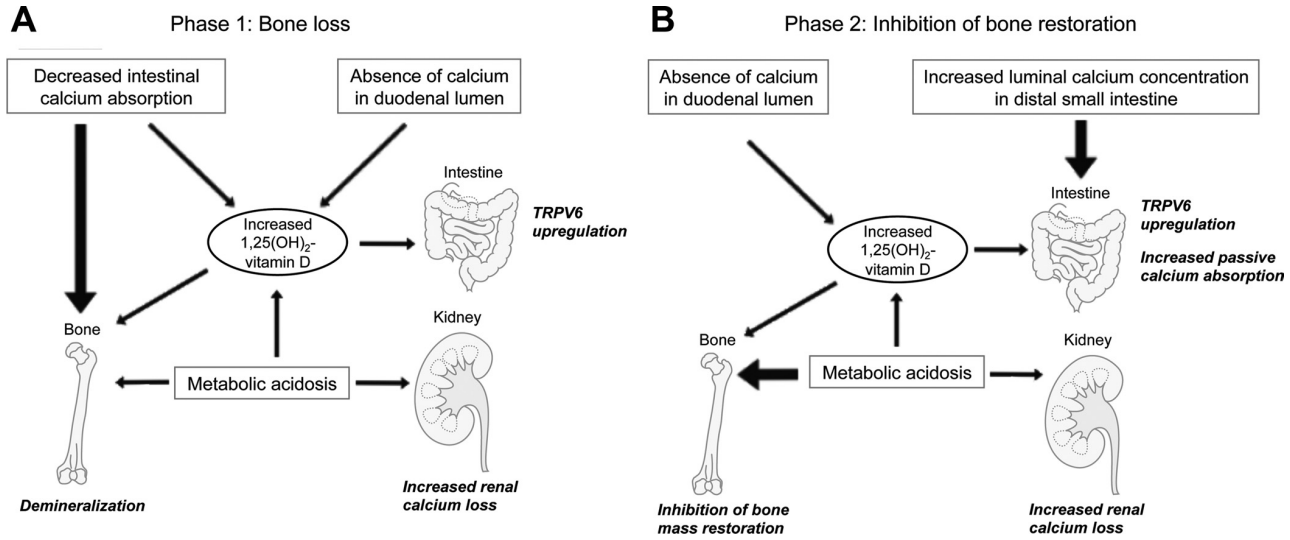

Fig. 8. Proposed mechanisms for bone loss and changes in calcium and vitamin D metabolism early after RYGB surgery (*A*) and after compensation of initial calcium malabsorption (*B*). *A*: active intestinal calcium absorption decreases early after Roux-en-Y gastric bypass surgery due to the exclusion of the duodenum from nutrient flow. Subsequently, increased conversion of 25-hydroxyvitamin D to active 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] may be further supported by metabolic acidosis and direct signaling from calcium-sensing enterocytes in the duodenum. Compensatory upregulation of the intestinal calcium channel TRPV6 by 1,25$(OH)_2$D is futile since TRPV6 is mainly expressed in the duodenum. Calcium mobilization from bones ensures stable blood calcium levels, which may be partly mediated by elevated 1,25$(OH)_2$D. In addition, metabolic acidosis potentially contributes to bone resorption and leads to decreased renal calcium reabsorption. *B*: after adaptation to the altered anatomy of the gastrointestinal tract, direct delivery of nutrients to the distal small intestine facilitates passive calcium absorption, which compensates for the decreased active absorption in the duodenum. The normalization of intestinal calcium malabsorption does not lead to a restoration of bone mass due to inhibitory effects of metabolic acidosis and potentially 1,25$(OH)_2$D on bone formation. Vitamin D activation and renal calcium loss remain increased. Thick arrows represent the proposed predominant mechanisms.

Second, bariatric surgery has become an important treatment option for morbid obesity in adolescents in recent years (36). Besides the loss of bone mass that has been described in adult patients, there may be additional negative effects of RYGB surgery on skeleton development during growth. 1,25$(OH)_2$D treatment causes altered maturation and narrowing of the epiphyseal growth plate in rats (23). The chronically elevated 1,25$(OH)_2$D levels detected in RYGB rats and also previously reported in adults after RYGB surgery could, therefore, have effects on the growing skeleton that may not be detected in adults.

Finally, we want to mention that there are some limitations of our study. Although our RYGB rat model has repeatedly been shown to mimic changes in physiology after RYGB surgery in humans, we sometimes find exaggerated responses to the surgery in animal models. Hence, the extent of postsurgical bone loss in humans and the question of whether there are relevant functional consequences may still be disputed, although recent evidence suggests a connection between RYGB surgery and fracture incidence (33). However, as already mentioned, the evaluation of changes in bone and calcium metabolism after RYGB surgery in humans is very challenging because of preexisting conditions, such as 25(OH)D deficiency and 2° HPT. In addition, most patients are supplemented with vitamin D and calcium after the surgery, but there is often no information about vitamin D and calcium levels prior to supplementation, there is no standardized replacement protocol, and information about compliance is difficult to obtain.

Another potential limitation of our study is that there are currently no data showing that RYGB surgery leads to metabolic acidosis in humans, even though cases of D-lactic acidosis have been described (7a). However, the most common calcium supplements, calcium citrate and calcium carbonate, also confer alkali load that could unintentionally prevent the development of an acidotic state; long-term assessment of acid-base imbalances in RYGB patients is, therefore, similarly challenging to the investigation of bone metabolism.

Lastly, we want to point out that the major advantages of using animal models such as ours are the close controllability of environmental factors, the standardization among groups, and the inclusion of different control groups, in particular, a group matched for body weight loss. We can, therefore, use the new evidence provided by experiments in animal models to improve and refine the design of studies involving human RYGB patients.

Perspectives and Significance

Our data point to a complex disruption of bone homeostasis after RYGB that is not a simple consequence of alterations in vitamin D and calcium levels. By the use of a body weight-matched control group, we demonstrated that chronic food restriction in sham-operated rats did not have comparable effects on bone metabolism. We showed that increased activation of vitamin D may compensate for intestinal vitamin D malabsorption in RYGB rats. Together with the absence of 2° HPT, this questions the role of vitamin D deficiency and 2° HPT in RYGB-induced bone loss. Interestingly, RYGB surgery led to increased lactate levels and chronic metabolic acidosis, which may have contributed to both the initial bone loss and the insufficient subsequent restoration of bone mass. Even though cases of D-lactic acidosis have been described in humans after RYGB surgery, there are no studies on postsurgical changes in acid-base status. Our data suggest that future studies should include consideration of metabolic acidosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.A., M.S., M.B., and T.A.L. conception and design of research; K.A. and N.G. performed experiments; K.A., N.G., and C.A.W. analyzed data; K.A., C.A.W., A.L., M.B., and T.A.L. interpreted results of experiments; K.A. prepared figures; K.A. drafted manuscript; K.A., C.A.W., A.L., M.S., M.B., and T.A.L. edited and revised manuscript; K.A., N.G., C.A.W., A.L., M.S., M.B., and T.A.L. approved final version of manuscript.

REFERENCES

1. **Aarts E, van Groningen L, Horst R, Telting D, van Sorge A, Janssen I, de Boer H.** Vitamin D absorption: consequences of gastric bypass surgery. *Eur J Endocrinol* 164: 827–832, 2011.
2. **Aasheim ET, Johnson LK, Hofso D, Bohmer T, Hjelmesaeth J.** Vitamin status after gastric bypass and lifestyle intervention: a comparative prospective study. *Surg Obes Relat Dis* 8: 169–175, 2012.
3. **Armbrecht HJ, Gross CJ, Zenser TV.** Effect of dietary calcium and phosphorus restriction on calcium and phosphorus balance in young and old rats. *Arch Biochem Biophys* 210: 179–185, 1981.
4. **Arnett TR.** Acidosis, hypoxia and bone. *Arch Biochem Biophys* 503: 103–109, 2010.
5. **Asarian L, Abegg K, Geary N, Schiesser M, Lutz TA, Bueter M.** Estradiol increases body weight loss and gut-peptide satiation after Roux-en-Y gastric bypass in ovariectomized rats. *Gastroenterology* 143: 325–327 322, 2012.
6. **Bal BS, Finelli FC, Shope TR, Koch TR.** Nutritional deficiencies after bariatric surgery. *Nat Rev Endocrinol* 8: 544–556, 2012.
7. **Berthelot M.** Violet d'aniline. *Rep Chim App* 1: 284, 1859.

7a. **Bhardwaj A, Watanabe M, Shah JR.** A 46-yr-old woman with ataxia and blurred vision 3 months after bariatric surgery. *Am J Gastroenterol* 103: 1575–1577, 2008.

8. **Bolt MJ, Liu W, Qiao G, Kong J, Zheng W, Krausz T, Cs-Szabo G, Sitrin MD, Li YC.** Critical role of vitamin D in sulfate homeostasis: regulation of the sodium-sulfate cotransporter by 1,25-dihydroxyvitamin D3. *Am J Physiol Endocrinol Metab* 287: E744–E749, 2004.
9. **Brandao-Burch A, Utting JC, Orriss IR, Arnett TR.** Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. *Calcif Tissue Int* 77: 167–174, 2005.
10. **Bueter M, Abegg K, Seyfried F, Lutz TA, and le Roux CW.** Roux-en-Y gastric bypass operation in rats. *J Vis Exp*: e3940, 2012.
11. **Bueter M, Lowenstein C, Olbers T, Wang M, Cluny NL, Bloom SR, Sharkey KA, Lutz TA, le Roux CW.** Gastric bypass increases energy expenditure in rats. *Gastroenterology* 138: 1845–1853, 2010.
12. **Carlin AM, Rao DS, Yager KM, Parikh NJ, Kapke A.** Treatment of vitamin D depletion after Roux-en-Y gastric bypass: a randomized prospective clinical trial. *Surg Obes Relat Dis* 5: 444–449, 2009.
13. **Casagrande DS, Repetto G, Mottin CC, Shah J, Pietrobon R, Worni M, Schaan BD.** Changes in bone mineral density in women following 1-year gastric bypass surgery. *Obes Surg* 22: 1287–1292, 2012.
14. **Clements RH, Yellumahanthi K, Wesley M, Ballem N, Bland KI.** Hyperparathyroidism and vitamin D deficiency after laparoscopic gastric bypass. *Am Surg* 74: 469–474; discussion 474–465, 2008.
15. **Doube M, Klosowski MM, Arganda-Carreras I, Cordelieres FP, Dougherty RP, Jackson JS, Schmid B, Hutchinson JR, Shefelbine SJ.** BoneJ: Free and extensible bone image analysis in ImageJ. *Bone* 47: 1076–1079, 2010.
16. **Earthman CP, Beckman LM, Masodkar K, Sibley SD.** The link between obesity and low circulating 25-hydroxyvitamin D concentrations: considerations and implications. *Int J Obes (Lond)* 36: 387–396, 2012.
17. **Egan KP, Brennan TA, Pignolo RJ.** Bone histomorphometry using free and commonly available software. *Histopathology* 61: 1168–1173, 2012.
18. **Erben RG, Glosmann M.** Histomorphometry in rodents. *Methods Mol Biol* 816: 279–303, 2012.
19. **Fleischer J, Stein EM, Bessler M, Della Badia M, Restuccia N, Olivero-Rivera L, McMahon DJ, Silverberg SJ.** The decline in hip bone density after gastric bypass surgery is associated with extent of weight loss. *J Clin Endocrinol Metab* 93: 3735–3740, 2008.
20. **Gassmann M, Grenacher B, Rohde B, Vogel J.** Quantifying Western blots: pitfalls of densitometry. *Electrophoresis* 30: 1845–1855, 2009.
21. **Grethen E, Hill KM, Jones R, Cacucci BM, Gupta CE, Acton A, Considine RV, Peacock M.** Serum leptin, parathyroid hormone, 1,25-dihydroxyvitamin D, fibroblast growth factor 23, bone alkaline phosphatase, and sclerostin relationships in obesity. *J Clin Endocrinol Metab* 97: 1655–1662, 2012.
22. **Hock JM, Gunness-Hey M, Poser J, Olson H, Bell NH, Raisz LG.** Stimulation of undermineralized matrix formation by 1,25 dihydroxyvitamin D3 in long bones of rats. *Calcif Tissue Int* 38: 79–86, 1986.
23. **Idelevich A, Kerschnitzki M, Shahar R, Monsonego-Ornan E.** 1,25(OH)2D3 alters growth plate maturation and bone architecture in young rats with normal renal function. *PLoS One* 6: e20772, 2011.
24. **Jehle S, Zanetti A, Muser J, Hulter HN, Krapf R.** Partial neutralization of the acidogenic Western diet with potassium citrate increases bone mass in postmenopausal women with osteopenia. *J Am Soc Nephrol* 17: 3213–3222, 2006.
25. **Jin J, Robinson AV, Hallowell PT, Jasper JJ, Stellato TA, Wilhem SM.** Increases in parathyroid hormone (PTH) after gastric bypass surgery appear to be of a secondary nature. *Surgery* 142: 914–920; discussion 914–920, 2007.
26. **Johnson JM, Maher JW, Samuel I, Heitshusen D, Doherty C, Downs RW.** Effects of gastric bypass procedures on bone mineral density, calcium, parathyroid hormone, and vitamin D. *J Gastrointest Surg* 9: 1106–1110; discussion 1110–1101, 2005.
27. **Krapf R, Vetsch R, Vetsch W, Hulter HN.** Chronic metabolic acidosis increases the serum concentration of 1,25-dihydroxyvitamin D in humans by stimulating its production rate. Critical role of acidosis-induced renal hypophosphatemia. *J Clin Invest* 90: 2456–2463, 1992.
28. **Lee GS, Jung EM, Choi KC, Oh GT, Jeung EB.** Compensatory induction of the TRPV6 channel in a calbindin-D9k knockout mouse: Its regulation by 1,25-hydroxyvitamin D3. *J Cell Biochem* 108: 1175–1183, 2009.
29. **Lieben L, Benn BS, Ajibade D, Stockmans I, Moermans K, Hediger MA, Peng JB, Christakos S, Bouillon R, Carmeliet G.** Trpv6 mediates intestinal calcium absorption during calcium restriction and contributes to bone homeostasis. *Bone* 47: 301–308, 2010.
30. **Lieben L, Masuyama R, Torrekens S, Van Looveren R, Schrooten J, Baatsen P, Lafage-Proust MH, Dresselaers T, Feng JQ, Bonewald LF, Meyer MB, Pike JW, Bouillon R, Carmeliet G.** Normocalcemia is maintained in mice under conditions of calcium malabsorption by vitamin D-induced inhibition of bone mineralization. *J Clin Invest* 122: 1803–1815, 2012.
31. **Lucioni A, Womack C, Musch MW, Rocha FL, Bookstein C, Chang EB.** Metabolic acidosis in rats increases intestinal NHE2 and NHE3 expression and function. *Am J Physiol Gastrointest Liver Physiol* 283: G51–G56, 2002.
32. **Miao D, He B, Lanske B, Bai XY, Tong XK, Hendy GN, Goltzman D, Karaplis AC.** Skeletal abnormalities in Pth-null mice are influenced by dietary calcium. *Endocrinology* 145: 2046–2053, 2004.
33. **Nakamura KM, Haglind EG, Clowes JA, Achenbach SJ, Atkinson EJ, Melton LJ, 3rd, Kennel KA.** Fracture risk following bariatric surgery: a population-based study. *Osteoporos Int* In press.
34. **Nijenhuis T, Renkema KY, Hoenderop JG, Bindels RJ.** Acid-base status determines the renal expression of Ca^{2+} and Mg^{2+} transport proteins. *J Am Soc Nephrol* 17: 617–626, 2006.
35. **Nowik M, Picard N, Stange G, Capuano P, Tenenhouse HS, Biber J, Murer H, Wagner CA.** Renal phosphaturia during metabolic acidosis revisited: molecular mechanisms for decreased renal phosphate reabsorption. *Pflügers Arch* 457: 539–549, 2008.
36. **Olbers T, Gronowitz E, Werling M, Marlid S, Flodmark CE, Peltonen M, Gothberg G, Karlsson J, Ekbom K, Sjostrom LV, Dahlgren J, Lonroth H, Friberg P, Marcus C.** Two-year outcome of laparoscopic

Roux-en-Y gastric bypass in adolescents with severe obesity: results from a Swedish Nationwide Study (AMOS). *Int J Obes (Lond)* 36: 1388–1395, 2012.

37. **Pan W, Borovac J, Spicer Z, Hoenderop JG, Bindels RJ, Shull GE, Doschak MR, Cordat E, Alexander RT.** The epithelial sodium/proton exchanger, NHE3, is necessary for renal and intestinal calcium (re)absorption. *Am J Physiol Renal Physiol* 302: F943–F956, 2012.
38. **Pansu D, Bellaton C, Roche C, Bronner F.** Duodenal and ileal calcium absorption in the rat and effects of vitamin D. *Am J Physiol Gastrointest Liver Physiol* 244: G695–G700, 1983.
39. **Pereira FA, de Castro JA, dos Santos JE, Foss MC, Paula FJ.** Impact of marked weight loss induced by bariatric surgery on bone mineral density and remodeling. *Braz J Med Biol Res* 40: 509–517, 2007.
40. **Petersen DC.** Lactic acidosis. *Nutr Clin Pract* 20: 634–645, 2005.
41. **Riedt CS, Brolin RE, Sherrell RM, Field MP, Shapses SA.** True fractional calcium absorption is decreased after Roux-en-Y gastric bypass surgery. *Obesity (Silver Spring)* 14: 1940–1948, 2006.
42. **Sernka TJ, Borle AB.** Calcium in the intestinal contents of rats on different calcium diets. *Proc Soc Exp Biol Med* 131: 1420–1423, 1969.
43. **Shah M, Simha V, Garg A.** Long-term impact of bariatric surgery on body weight, comorbidities, and nutritional status. *J Clin Endocrinol Metab* 91: 4223–4231, 2006.
44. **Sinha N, Shieh A, Stein EM, Strain G, Schulman A, Pomp A, Gagner M, Dakin G, Christos P, Bockman RS.** Increased PTH and 1.25(OH)(2)D levels associated with increased markers of bone turnover following bariatric surgery. *Obesity (Silver Spring)* 19: 2388–2393, 2011.
45. **Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC.** Measurement of protein using bicinchoninic acid. *Anal Biochem* 150: 76–85, 1985.
46. **Soleymani T, Tejavanija S, Morgan S.** Obesity, bariatric surgery, and bone. *Curr Opin Rheumatol* 23: 396–405, 2011.
47. **Stemmer K, Bielohuby M, Grayson BE, Begg DP, Chambers AP, Neff C, Woods SC, Erben RG, Tschop MH, Bidlingmaier M, Clemens TL, Seeley RJ.** Roux-en-Y gastric bypass surgery but not vertical sleeve gastrectomy decreases bone mass in male rats. *Endocrinology* 154: 2015–2024, 2013.
48. **Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van Herck E, Kato S, Bindels RJ, Collen D, Carmeliet P, Bouillon R, Carmeliet G.** Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci USA* 98: 13324–13329, 2001.
49. **Viegas M, Vasconcelos RS, Neves AP, Diniz ET, Bandeira F.** Bariatric surgery and bone metabolism: a systematic review. *Arq Bras Endocrinol Metabol* 54: 158–163, 2010.
50. **Vilarrasa N, San Jose P, Garcia I, Gomez-Vaquero C, Miras PM, de Gordejuela AG, Masdevall C, Pujol J, Soler J, Gomez JM.** Evaluation of bone mineral density loss in morbidly obese women after gastric bypass: 3-year follow-up. *Obes Surg* 21: 465–472, 2011.
51. **Wang Y, Zhu J, DeLuca HF.** Where is the vitamin D receptor? *Arch Biochem Biophys* 523: 123–133, 2012.
52. **Woudenberg-Vrenken TE, Lameris AL, Weissgerber P, Olausson J, Flockerzi V, Bindels RJ, Freichel M, Hoenderop JG.** Functional TRPV6 channels are crucial for transepithelial Ca^{2+} absorption. *Am J Physiol Gastrointest Liver Physiol* 303: G879–G885, 2012.
53. **Xi Q, Wang S, Ye Z, Liu J, Yu X, Zhu Z, Su S, Bai J, Li C.** Adenovirus-delivered microRNA targeting the vitamin D receptor reduces intracellular Ca^{2+} concentrations by regulating the expression of Ca^{2+}-transport proteins in renal epithelial cells. *BJU Int* 107: 1314–1319, 2011.
54. **Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, Parameswaran P, Crowell MD, Wing R, Rittmann BE, Krajmalnik-Brown R.** Human gut microbiota in obesity and after gastric bypass. *Proc Natl Acad Sci USA* 106: 2365–2370, 2009.

6. Original Research Article: “Acute peripheral GLP-1 Receptor Agonism or Antagonism does not alter Energy Expenditure in Rats after Roux-en-Y Gastric Bypass “

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My contribution to this manuscript includes the study design, data acquisition, data analysis, data interpretation and drafting and revising the manuscript.

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Acute peripheral GLP-1 receptor agonism or antagonism does not alter energy expenditure in rats after Roux-en-Y gastric bypass☆

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HIGHLIGHTS

- Compensatory fall in energy expenditure after weight loss is attenuated by RYGB.
- Acute injection of GLP-1 antagonist Exendin-9 increases eating only in RYGB rats.
- Enhanced release of GLP-1 may contribute to inhibitory effect of RYGB on eating.
- Acute administration of GLP-1 agonist Exendin-4 decreases eating more in RYGB rats.
- Acute modulation of GLP-1 signaling does not alter energy expenditure in RYGB rats.

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ABSTRACT

Compared to traditional weight loss strategies, the compensatory decrease in energy expenditure in response to body weight loss is markedly attenuated after Roux-en-Y gastric bypass surgery (RYGB). Because basal and post-prandial levels of glucagon-like peptide-1 (GLP-1) are increased after RYGB surgery, and because GLP-1 has been shown to increase energy expenditure, we investigated if increased GLP-1 levels are involved in the alterations in energy expenditure after RYGB. Adult male Wistar rats were randomized for RYGB (n = 8) or sham surgery (n = 17). Part of the sham-operated rats were food restricted and body weight-matched (n = 8) to the RYGB animals. The effects of acute subcutaneous administration of the GLP-1 antagonist Exendin (9–39) (Ex-9, 30 μg/kg) or the GLP-1 agonist Exendin-4 (Ex-4, 5 μg/kg), respectively, on energy expenditure were tested using indirect calorimetry. We found that Ex-9 increased food intake in RYGB, but not in sham-operated rats. Energy expenditure was lower in RYGB and sham-operated body weight-matched rats compared to sham-operated ad libitum fed rats, but significantly higher in RYGB rats compared to sham-operated body weight-matched rats. There was no effect of Ex-9 treatment on energy expenditure in either group of animals. Similarly, Ex-4 decreased food intake more in RYGB than in sham-operated rats, but Ex-4 did not modulate energy expenditure in any surgical group. We conclude that acute modulation of GLP-1 signaling is not directly involved in altered energy expenditure after RYGB surgery in rats.

1. Introduction

Obesity with its resulting comorbidities has become a major topic in global healthcare and disease prevention [1]. Currently, bariatric surgery is the treatment of choice for obese patients because weight loss maintenance and improvement or even resolution of co-morbidities such as type 2 diabetes mellitus is achieved in many cases [2–5]. The Roux-en-Y Gastric Bypass (RYGB) is the most commonly performed bariatric procedure and can be considered the gold standard in bariatric surgery [6]. Several underlying physiological mechanisms have been identified that potentially contribute to weight loss after RYGB; these mechanisms include reduced hunger and increased satiation [7–9], changes in meal patterns [10], a reduced preference for high fat diet [11–13], alterations in sweet taste function [12–15], as well as absence of a compensatory decrease in energy expenditure [16,17].

We and others previously reported that body weight loss in rats after RYGB is not associated with the decrease in energy expenditure that is observed with traditional weight loss strategies [16,17]. This finding was interesting because maintenance of a lower body weight by dietary caloric restriction fails in many obese patients due to compensatory metabolic responses such as a decrease of energy expenditure [18]. The reasons for the absence of decreased energy

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expenditure after RYGB surgery in rats are unknown, but it has been hypothesized that the increased postprandial release of glucagon-like peptide-1 from the distal small intestine after RYGB may be involved [7,8,19]. From all gastrointestinal hormones affected by RYGB, GLP-1 has most consistently been reported to be elevated after RYGB in humans and rats and is thought to be at least partly responsible for the reduction in food intake and the early improvement of glucose tolerance after surgery [4,5,7,8,19,20].

GLP-1's effects on food intake have been characterized in numerous studies (e.g., [21–25]), but its role in the control of energy expenditure is less well investigated. However, there are some studies that suggest an involvement of GLP-1 in energy expenditure. For example, Osaka et al. showed a dose-dependent increase in oxygen consumption after intravenous GLP-1 administration [26]. Furthermore, mice lacking the GLP-1 degrading enzyme dipeptidyl peptidase IV (DPP IV) are resistant to high fat diet-induced obesity due to reduced food intake and increased energy expenditure [27].

In this project, we therefore wanted to assess a possible role of the endocrine system and in particular of the satiating gut hormone GLP-1 in energy expenditure after RYGB in rats. We hypothesized that acute peripheral modification of GLP-1 signaling may influence the changes of energy expenditure induced by RYGB.

2. Methods

2.1. Animals

Twenty-five adult male Wistar rats weighing 400–450 g preoperatively were allocated to either RYGB ($n = 8$) or sham-operation ($n = 17$). After a recovery period of 10 days, sham-operated animals were randomly divided into two groups: sham-operated rats with no dietary manipulation ($n = 9$, ad libitum fed shams weighing 441 ± 16 g 10 days after surgery) and food-restricted shams whose postoperative weight was matched to the weight of RYGB rats (body weight-matched shams (BWm) weighing 433 ± 20 g 10 days after surgery). BWm shams received as much food daily as was necessary to maintain a similar body weight as the RYGB rats. Based on experiences from previous studies [16], rats were given approximately 14 g of standard chow in the beginning of the food restriction period. This amount of food was offered at dark onset and readjusted every third day depending on the body weight development.

2.2. Housing

All animals were individually housed under an artificial 12 hour/12 hour light–dark cycle (lights off at 1500) and at a room temperature of 21 ± 2 °C unless otherwise stated. Water and standard chow were available ad libitum. All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland.

2.3. Surgery

Anesthesia was induced in a chamber filled with 5% isoflurane in oxygen (1 l/min). After an adequate depth of anesthesia was achieved, rats were shaved from sternum to pelvis followed by disinfection with betadine scrub. Rats were then placed in supine position on a heating pad and positioned in a nose cone to maintain anesthesia (2–4% isoflurane in oxygen, 0.5 l/min) for the duration of surgery. Operations were performed as previously described [28]. Briefly, a midline incision of approximately 4 cm starting just below the xyphoid process was performed. For the RYGB procedure, the small bowel was transected approximately 20 cm distal to the pylorus of the stomach creating a proximal and distal end of small bowel. The proximal end being still continuous with the remaining portion of the stomach constituted the biliopancreatic limb and was anastomosed to the ileum approximately 25–30 cm from the caecum creating the common channel. For formation of the gastric pouch, the stomach was transected approximately 5 mm below the gastro-esophageal junction creating a gastric pouch of a size of no more than 2–3% of original stomach size. The Roux-en-Y reconstruction was completed by connecting the distal end of the small bowel to the gastric pouch leading to formation of the alimentary limb. One single RYGB procedure lasted approximately 70 min. For sham operations, an anterior gastrostomy and a jejunostomy with subsequent closures were performed. One single sham procedure lasted approximately 30 min. The abdominal wall and the skin were closed in layers after both operations.

2.4. Indirect calorimetry

Measurements were conducted in an open circuit calorimetry system (AccuScan Inc., USA) as previously described [29,30]. Briefly, rats were individually housed in Plexiglas airtight metabolic cages (41 × 41 × 31 cm) on a layer of wood shavings under the same light and temperature conditions as described above. Water and standard powder chow (GLP3433, Provimi Kliba Ag, Switzerland) were available ad libitum, unless otherwise stated. Food and water intakes were measured continuously. Physical activity was monitored by a 3-dimensional array of infrared light beams and sensors. Thus, the activity data provided represent a relative measure of locomotor activity of the rats (movement/hour). The activity data do not relate to an absolute measurement of distance moved or to a spatial position.

Energy expenditure was calculated for each 30 second sample according to Weir using the following equitation: total energy expenditure (kcal/h) = $3.9 \times V(O_2)L / h + 1.1 \times V(CO_2)L / h$ [29,30]. The respiratory quotient was defined as the quotient of CO_2 production and O_2 consumption.

2.5. Experimental design

Measurements of energy expenditure in all groups were conducted between postoperative weeks 5 and 11. Experiments were performed in a randomized, saline-controlled, crossover manner. About one week before treatment, all rats were placed for at least two days in the metabolic chambers for adaptation.

First, the potency of endogenous GLP-1 to alter energy expenditure after RYGB was tested with the GLP-1 receptor antagonist Exendin (9–39) (Ex-9); we allowed two days of wash-out between crossover days, i.e. between peptide and saline injections, respectively. Tests were performed similar to Williams et al. [25]. As shown in Fig. 1, rats were deprived of chow, but not water, 8 h prior to dark onset (0700 h). At 1100 h, 30 μg/kg Ex-9 or the saline vehicle (1 ml/kg) was injected subcutaneously and measurement of energy expenditure was started. One hour later chow was provided ad libitum again (1200 h, i.e. 3 h prior to dark onset).

Second, the potency of exogenous GLP-1 to alter energy expenditure after RYGB was tested with the GLP-1 agonist Exendin-4 (Ex-4) with three days of wash-out between peptide and saline. As demonstrated in Fig. 2, rats were deprived of chow, but not water, 6 h prior to dark onset (0900 h). At 1400 h, 5 μg/kg Ex-4 or the saline vehicle (1 ml/kg) was injected subcutaneously and measurement of energy expenditure was started. One hour later, i.e. at dark onset, chow was provided ad libitum again (1500 h). The experiments using Ex-4 were performed about 4 weeks after the experiments using Ex-9. Dose and time course of Ex-4 administration were chosen based on an unpublished pilot experiment that showed a significant decrease in chow intake of intact male Wistar rats ($n = 12$) up to 24 h after injection.

2.6. Statistical analysis

A one-way ANOVA was performed to analyze average daily food intake as well as differences in body weight between groups. Following a

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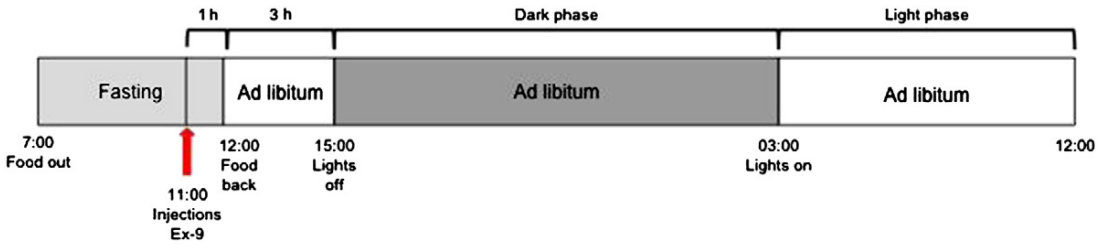

Fig. 1. Schematic illustration of the experimental protocol to analyze the treatment effect of GLP-1 receptor antagonist Exendin (9–39). Rats were deprived of food 8 h prior to dark onset (0700 h). At 1100 h, 30 μg/kg Ex-9 or the saline vehicle (1 ml/kg) was injected subcutaneously. One hour later chow was provided ad libitum again (1200 h).

significant F ratio, a Bonferroni post hoc test was used to determine differences between groups. Differences in energy expenditure, food intake, physical activity and respiratory quotients after Ex-9 and Ex-4 treatments were analyzed with a two-way, repeated measures (RM) group (between subjects) × treatment (within subjects) analysis of variance (ANOVA) followed by Bonferroni post-hoc tests for each treatment group when there was a significant group × treatment interaction. Significance was established at $p \leq 0.05$ for all statistical sets and data are reported as mean ± SEM.

3. Results

3.1. Body weight

Fig. 3 shows the development of body weight for all three groups. Body weight was significantly lower in RYGB rats compared to the sham-operated ad libitum fed group from postoperative day 6 (sham ad lib: 441 ± 16 g vs. RYGB: 383 ± 16 g, $p = 0.020$). In postoperative week 14, the difference in body weight was about 240 g (sham ad lib: 628 ± 34 g vs. RYGB: 389 ± 81 g, $p = 0.012$). After a short period of post-surgical weight loss, shams ad libitum fed constantly gained weight for the rest of the study. In contrast, RYGB animals lost 20% of their preoperative weight by postoperative week 2 with a weight nadir of 360 ± 45 g. Until postoperative week 5, RYGB rats then regained a moderate amount of body weight followed by a plateau around 380 and 390 g throughout the rest of the observation period. Food restriction started ten days after surgery for sham-operated BWm rats ($n = 8$). There was no significant difference in body weight between the RYGB group and the food restricted sham BWm rats at postoperative week 4 (sham BWm: 405 ± 13 g vs. RYGB: 379 ± 43, $p = 0.56$) and thereafter.

3.2. Average daily food intake

Fig. 4 shows the average daily food intake for all three groups throughout the entire observation period from postoperative weeks 0–14. Daily food intake was consistently lower after RYGB (sham ad lib: 29.6 ± 0.9 g vs. RYGB: 25.5 ± 0.8 g, $p < 0.01$). BWm shams required significantly less food than RYGB animals to maintain the same level of body weight (sham BWm: 14.4 ± 0.04 g vs. RYGB: 25.5 ± 0.8 g, $p < 0.001$).

3.3. Effect of treatment with GLP-1 receptor antagonist Exendin (9–39)

3.3.1. Energy expenditure

Repeated measures (RM) two-way ANOVA revealed a main effect of surgical group for mean 24 hour energy expenditure ($p < 0.001$); energy expenditure after RYGB was significantly higher compared to sham-operated BWm controls, but significantly lower in comparison to sham-operated ad libitum fed rats after both saline and Ex-9 treatments (Fig. 5A). However, there was no difference in 24 hour energy expenditure when comparing the corresponding surgery groups that were treated with saline or Ex-9, respectively; RM two-way ANOVA showed no main effect of treatment for saline or Ex-9 treatment, respectively ($p = 0.79$). There was also no significant group × treatment interaction ($p = 0.72$).

During the first hour after Ex-9 and saline injection with no food available (1100–1200), RM two-way ANOVA revealed a main effect of surgical group ($p < 0.001$). Energy expenditure tended to be higher after RYGB in comparison to sham-operated BWm controls, but this did not reach statistical significance. However, energy expenditure after RYGB was significantly lower than after sham-operation and ad libitum food; this was observed after both saline and Ex-9 treatments, respectively (Fig. 5B). Similar to the 24 h values, Ex-9 had no effect on energy expenditure during the first hour after treatment in any surgery group, i.e. there was no main effect of treatment for saline or Ex-9, respectively ($p = 0.88$). There was also no significant group × treatment interaction ($p = 0.58$).

For the first 2 h after food was made available ad libitum again (1200–1400), RM two-way ANOVA showed no main effect of surgical group ($p = 0.32$) or treatment ($p = 0.87$) for energy expenditure after Ex-9 and saline injections, respectively; there was also no group × treatment interaction ($p = 0.72$) (Fig. 5C). Fig. 5D shows the time course of the 24 hour energy expenditure after Ex-9 and saline treatment for all three groups. The values of the RM two-way ANOVA for energy expenditure after Ex-9 treatment are given in Table 1.

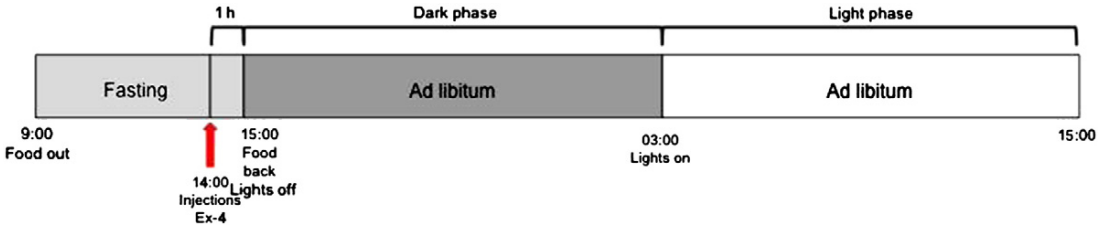

Fig. 2. Schematic illustration of the experimental protocol to analyze the treatment effect of GLP-1 receptor antagonist Exendin-4. Rats were deprived of food 6 h prior to dark onset (0900 h). At 1400 h, 5 μg/kg Ex-4 or the saline vehicle (1 ml/kg) was injected subcutaneously. One hour later, chow was provided ad libitum again (1500 h).

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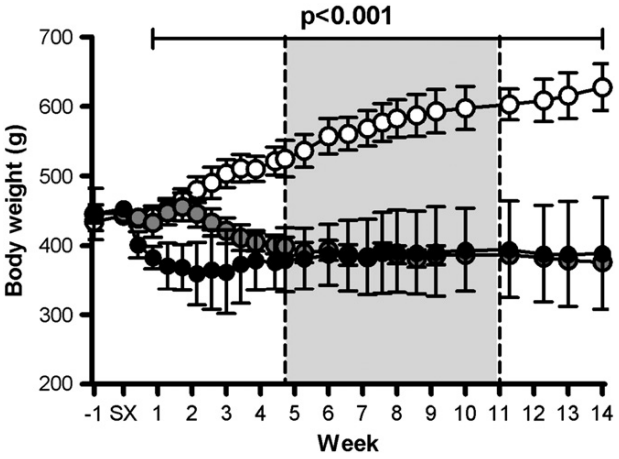

Fig. 3. Body weight change for the sham-operated rats ad libitum fed (white circles, n = 9), sham-operated BWm rats (gray circles, n = 8) and for RYGB rats (black circles, n = 8). E Body weight was significantly lower in RYGB rats compared to the sham-operated ad libitum fed group from postoperative day 6 as indicated ($p < 0.001$). Energy expenditure measurements were performed between postoperative week 5 and 11as indicated. Data are shown as mean values ± SEM.

3.3.2. Food intake, physical activity and respiratory quotient (RQ)

Food intake was analyzed for the first 2 h once food was made available ad libitum again (1200–1400). Here, RM two-way ANOVA showed a main effect of surgical group for mean food intake after Ex-9 and saline injections, respectively ($p < 0.001$) (Fig. 6A). While there was no main effect of treatment for mean food intake ($p = 0.13$), RM two-way ANOVA revealed a significant group × treatment interaction for mean food intake after Ex-9 or saline injection ($p = 0.004$), respectively, indicating that the Ex-9 effect differed significantly between the surgical groups. RYGB rats, but not sham-operated rats ate significantly more after Ex-9 than after saline (RYGB: saline 3.5 ± 0.4 g vs. Ex-9 4.9 ± 0.6 g, $p < 0.01$); in fact, sham-operated rats ate about 25% less after Ex-9 compared to saline, and RYGB rats ate about 65% more after Ex-9 during the first 2 h of food access (Fig. 6B; sham: 73.7 ± 13.2% vs. RYGB: 165.1 ± 25.3%, $p < 0.05$). Further, sham-operated BWm rats showed the highest food intake in this two hour period, which is clearly the result of being conditioned to a feeding regime with long periods of fasting before a limited amount of food was offered at dark onset. Thus, it was not surprising that we did not observe an effect of Ex-9 treatment in this group of rats.

Repeated measures (RM) two-way ANOVA further revealed a main effect of surgical group for the physical activity during the first 2 h after food return ($p < 0.001$), with sham-operated BWm controls showing more movements per hour than sham-operated ad libitum fed or RYGB operated rats (Fig. 6C). However, there was no main effect for treatment ($p = 0.87$) and no significant group × treatment interaction ($p = 0.72$). Finally, RM two-way ANOVA showed a significant main effect of surgical group for the respiratory quotient during the first 2 h after food return ($p < 0.05$), but there was no main effect for treatment and no group × treatment interaction (Fig. 6D). Sham-operated BWm controls had significantly higher respiratory quotients than sham-operated ad libitum fed and RYGB operated rats, irrespective of Ex-9 or saline injection. The p values of the RM two-way ANOVA for food intake, physical activity and respiratory quotient after Ex-9 treatment are given in Table 1.

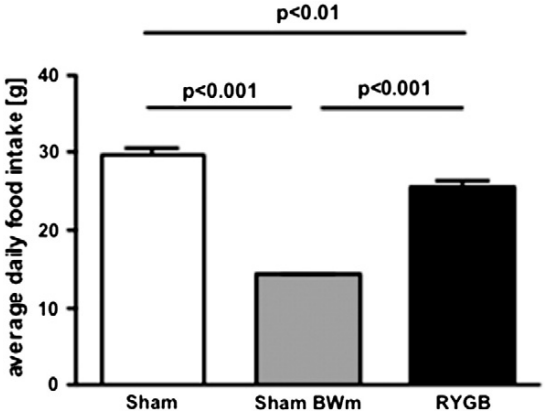

Fig. 4. Average daily food intake over 14 weeks for sham-operated ad libitum fed rats (n = 9, white column), for sham-operated BWm rats (n = 8, gray column) and for RYGB rats (n = 8, black column). Data are shown as mean values ± SEM.

3.4. Effect of GLP-1 receptor agonist Exendin-4

3.4.1. Energy expenditure

Repeated measures (RM) two-way ANOVA revealed a main effect of surgical group for mean 24 hour energy expenditure ($p < 0.001$) with energy expenditure after RYGB being significantly higher compared to sham-operated BWm controls, but significantly lower in comparison to sham-operated ad libitum fed rats after both saline and Ex-4 treatments (Fig. 7A). However, there was no Ex-4 effect in any surgical group; RM two-way ANOVA showed no main effect of treatment for saline or Ex-4, respectively ($p = 0.69$) and no significant group × treatment interaction ($p = 0.83$).

Similar to the 24 h values, RM two-way ANOVA revealed a main effect of surgical group on energy expenditure during the first hour after Ex-4 and saline when no food was available (1400–1500) ($p < 0.001$). Energy expenditure tended to be higher after RYGB compared to sham-operated BWm controls after saline or Ex-4, respectively ($p > 0.05$), but lower than after sham-operation and ad libitum food (Fig. 7B).

Overall, we found a main effect of treatment during the first hour after Ex-4 and saline, respectively, when rats were fasted ($p < 0.001$); Ex-4 reduced energy expenditure in all surgical groups of rats, but differences were only significant in sham-operated BWm rats (sham: saline: 2.5 ± 0.2 kcal/h vs. Ex-4: 2.4 ± 0.1 kcal/h, $p > 0.05$; sham BWm: saline: 1.9 ± 0.2 kcal/h vs. Ex-4: 1.5 ± 0.03 kcal/h, $p < 0.01$; RYGB: saline: 2.1 ± 0.1 kcal/h vs. 1.9 ± 0.1 kcal/h, $p > 0.05$). There was no significant group × treatment interaction ($p = 0.17$).

Repeated measures (RM) two-way ANOVA further showed a main effect of surgical group ($p < 0.001$), but not treatment ($p = 0.16$), on energy expenditure during the dark phase after Ex-4 and saline injections (Fig. 7C), respectively, but there was also no group × treatment interaction ($p = 0.48$). Similar to the complete 24 h period, energy expenditure was higher after RYGB than in sham-operated BWm controls during the dark phase, but significantly lower in comparison to sham-operated ad libitum fed rats after both saline and Ex-4 treatments. Fig. 7D shows the time course of the 24 hour energy expenditure after Ex-4 and saline treatment for all three surgical groups. The values of the two-way ANOVA for energy expenditure after Ex-4 treatment are given in Table 2.

3.4.2. Food intake, physical activity and respiratory quotient (RQ)

Repeated measures (RM) two-way ANOVA showed a main effect of the surgical group for mean food intake during the dark phase following Ex-4 or saline injection, respectively ($p < 0.001$) (Fig. 8A). There was also a main effect of treatment for mean food intake ($p < 0.001$) and a significant group × treatment interaction ($p < 0.001$). Sham-operated and RYGB rats reduced their food intake after Ex-4 administration (sham: saline: 25.3 ± 0.5 g vs. Ex-4: 20.8 ± 0.8 g, $p < 0.001$; RYGB: saline: 18.9 ± 2.2 g vs. Ex-4: 11.6 ± 1.2 g, $p = 0.004$). The Ex-4 induced reduction in dark phase eating was significantly stronger in RYGB than in sham-operated rats (Fig. 8B [saline control = 100%]; sham: 82.3 ± 2.3% vs. RYGB: 64.4 ± 5.9%, $p = 0.008$).

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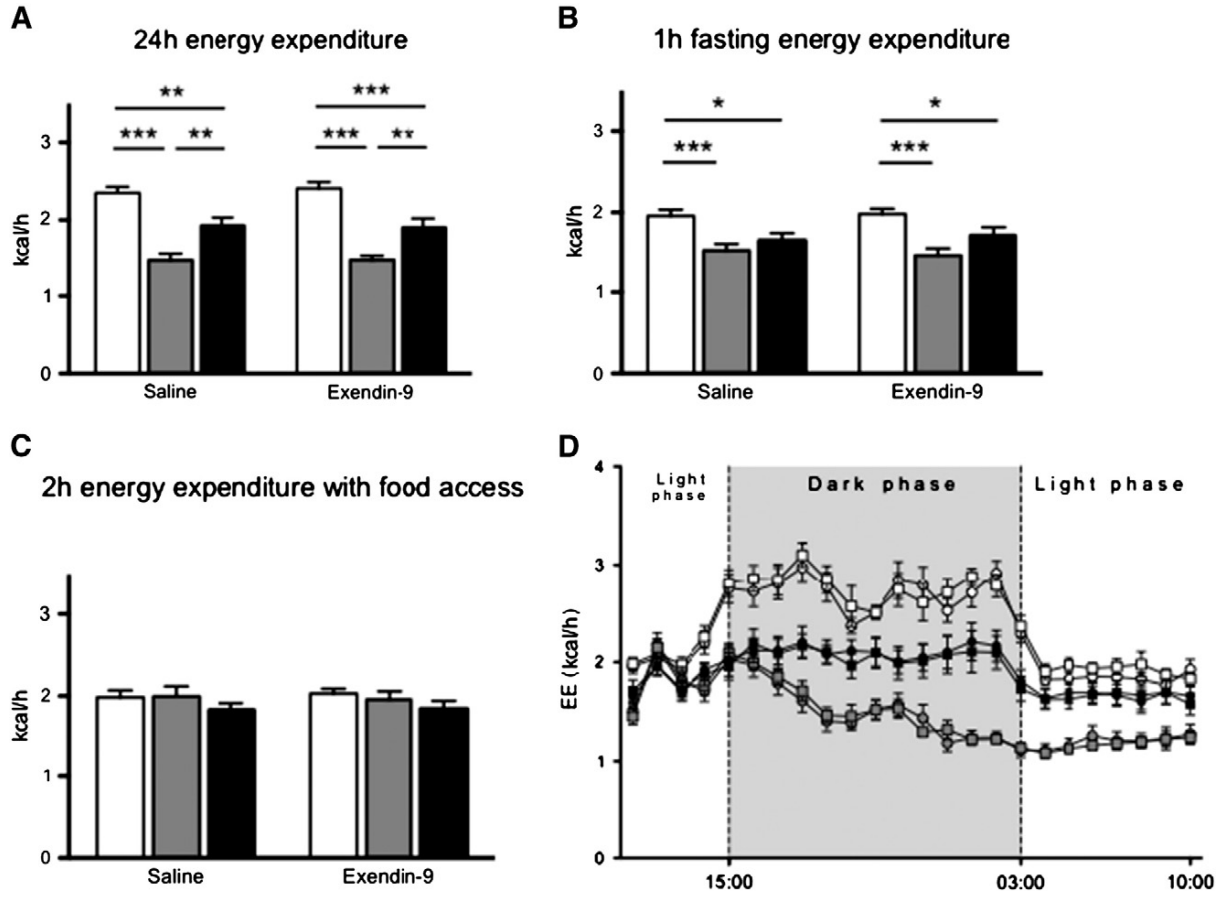

Fig. 5. A. Mean 24 h energy expenditure. B. Average energy expenditure during the first hour after Exendin (9–39) (30 μg/kg) and saline s.c. administration without food available. C. Average energy expenditure during the first 2 h after food return after Exendin (9–39) (30 μg/kg) and saline s.c. administration. D. Time course of 24 h energy expenditure after Exendin (9–39) (30 μg/kg) and saline s.c. administration. In A, B and C sham-operated rats ad libitum fed are shown by white columns, sham-operated BWm rats by gray columns and RYGB rats by black columns. In D a similar color code is used with squares representing Exendin (9–39) and circles representing saline treated rats. When two-way ANOVA revealed a significant F ratio, a Bonferroni post hoc test was used to determine differences between groups (*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$). Data are shown as mean values ± SEM.

Repeated measures (RM) two-way ANOVA further revealed no significant differences in physical activity and respiratory quotient between the three groups during the dark phase after Ex-4 and saline injections, respectively (Fig. 8C and D). However, there was a main effect of treatment during the first hour after Ex-4 and saline, respectively, when rats were fasted ($p < 0.01$); Ex-4 reduced activity in all surgical groups of rats, but differences were only significant in sham-operated BWm rats (sham: saline: 86.78 ± 18.6 movements/h vs. Ex-4: 54.0 ± 6.0 movements/h, $p > 0.05$; sham BWm: saline: 171.8 ± 34.5 movements/h vs. Ex-4: 64.4 ± 10.4 movements/h, $p < 0.05$; RYGB: saline: 125.5 ± 32.4 movements/h vs. 61.1 ± 11.4 movements/h, $p > 0.05$). The values of the RM two-way ANOVA for food intake, physical activity and respiratory quotient after Ex-4 treatment are given in Table 2.

4. Discussion

We demonstrated that acute subcutaneous administration of a low dose of the GLP-1 antagonist Exendin (9–39) (Ex-9, 30 μg/kg) or the GLP-1 agonist Exendin-4 (Ex-4, 5 μg/kg) did not alter energy expenditure in RYGB or sham-operated rats. Overall energy expenditure was lower in RYGB compared to sham-operated ad libitum fed rats, but energy expenditure was significantly higher in RYGB rats compared to their body weight matched counterparts. Against our hypothesis, we

Table 1
RM two-way ANOVA values for energy expenditure, food intake, physical activity and respiratory quotient during 24 h or the first 3 h and after Ex-9 or saline treatment, respectively, as a function of surgical group treatment.

	Surgical group	Treatment group	Interaction
24 h energy expenditure	$F(2,22) = 32.8$, $p < 0.001$	$F(1,22) = 0.7$, $p = 0.79$	$F(2,22) = 0.34$, $p = 0.72$
1 h energy expenditure (1100–1200, no food)	$F(2,22) = 11.74$, $p < 0.001$	$F(1,22) = 0.02$, $p = 0.88$	$F(2,22) = 0.34$, $p = 0.58$
2 h energy expenditure (1200–1400, with food)	$F(2,22) = 1.21$, $p = 0.32$	$F(1,22) = 0.03$, $p = 0.87$	$F(2,22) = 0.34$, $p = 0.72$
2 h food intake (1200–1400)	$F(2,21) = 457.3$, $p < 0.001$	$F(1,21) = 2.47$, $p = 0.13$	$F(2,21) = 7.46$, $p = 0.004$
2 h physical activity (1200–1400, with food)	$F(2,21) = 36.8$, $p < 0.001$	$F(1,21) = 0.03$, $p = 0.87$	$F(2,21) = 0.34$, $p = 0.72$
2 h respiratory quotient (1200–1400, with food)	$F(2,21) = 5.3$, $p = 0.013$	$F(1,21) = 0.15$, $p = 0.71$	$F(2,21) = 1.35$, $p = 0.28$

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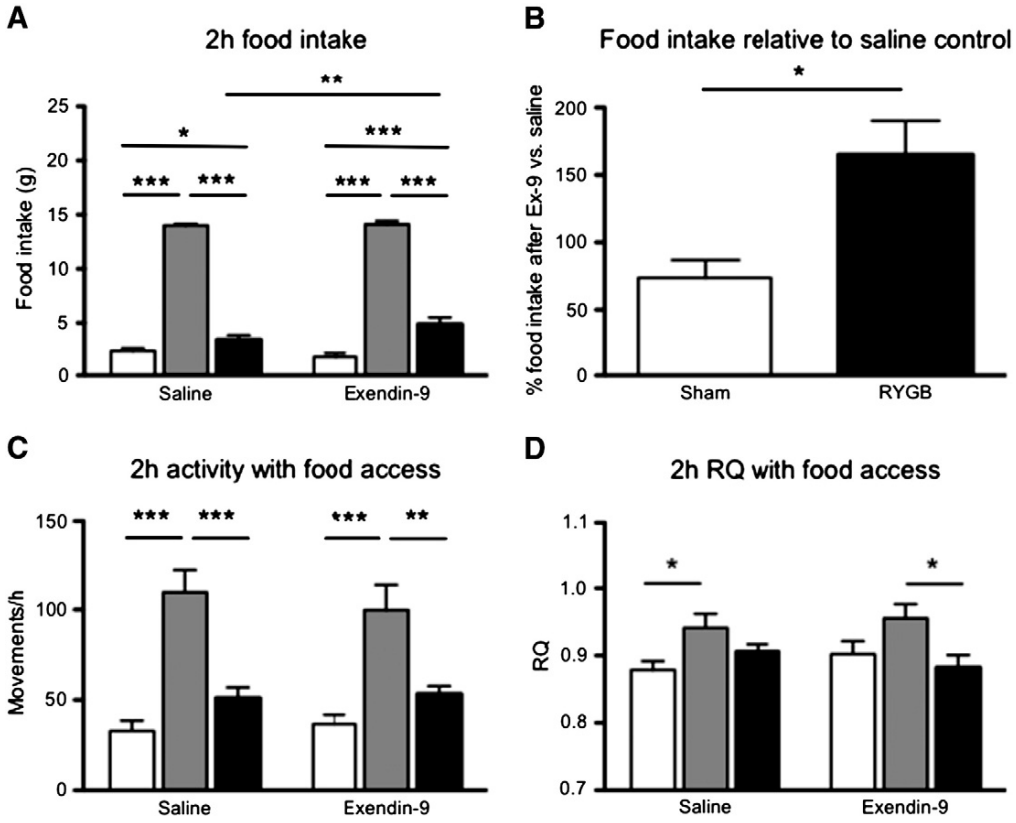

Fig. 6. A. Average spontaneous food intake during the first 2 h when food was available. B. Percentage change in 2 h food intake after Ex-9 treatment compared to saline treatment. C. Average physical activity during the first 2 h after food return D Average Respiratory Quotient during the first 2 h after food return for sham-operated rats ad libitum fed (white columns), sham-operated BWm rats (gray columns) and RYGB rats (black columns). As two-way ANOVA revealed a significant F ratio, a Bonferroni post hoc test was used to determine differences between groups (*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$). Data are shown as mean values ± SEM.

found that the administration of Ex-9 or Ex-4, respectively, at doses that affected eating had no measurable effect on energy expenditure in either group of animals.

Consistent with this study, we and others had previously reported that body weight loss in rats after RYGB was not associated with the decrease in energy expenditure that is usually observed with traditional weight loss strategies [16,17,31,32]. Such compensatory metabolic responses with a decrease in the resting metabolic rate are believed to be the main reason why maintenance of a lower body weight only by caloric restriction fails in the majority of obese patients [18]. The lack of this compensatory decrease in energy expenditure is therefore an important and promising finding that requires further investigation in order to detect the underlying mechanisms.

We and others had reported that the RYGB procedure leads to an increased postprandial release of GLP-1 in humans and rats. GLP-1's effects on food intake have been characterized in numerous studies (e.g., [21,23–25,33]), and elevated GLP-1 is thought to be at least partly responsible for the reduction in food intake after RYGB surgery. Several studies point to an additional role of GLP-1 in the regulation of energy balance by directly influencing energy expenditure. For example, Osaka et al. [26] showed a dose-dependent increase in oxygen consumption after intravenous GLP-1 administration; this effect was thought to be mediated by the lower brainstem and to require the integrity of the sympathoadrenal system. In addition, Lockie et al. [34] reported an increase in brown adipose tissue (BAT) thermogenesis after acute central injection of GLP-1, which was associated with increased activity of sympathetic fibers that innervate BAT. Furthermore, mice lacking the GLP-1 degrading enzyme dipeptidyl peptidase IV (DPP IV) are resistant to high fat diet-induced obesity because of reduced food intake and increased energy expenditure [27]. However, the latter results have to be interpreted with caution because DPP IV not only degrades GLP-1, but is also involved in the metabolism of many other peptides including PYY (which is activated to PYY3–36), oxyntomodulin (OXM) and glucagon-like peptide-2 (GLP-2). Like GLP-1, postprandial levels of these hormones are increased after RYGB surgery [8,19,35–37], and several studies show an involvement of PYY [38] and OXM [39,40] in the control of energy expenditure.

In the present study, we found no evidence that acute modulation of GLP-1 signaling affects the changes in energy expenditure seen after RYGB, even though we saw the expected effects of Ex-9 (increase) and Ex-4 (decrease) on eating. In fact, we provide evidence that at least under our experimental conditions enhanced GLP-1 signaling contributes to the eating inhibitory effect of RYGB, because Ex-9 increased eating only in RYGB but not in sham-operated rats. We also showed that Ex-4 decreased eating more in RYGB than in sham-operated rats. Hence, despite higher baseline and postprandial GLP-1 levels [19,41,42], RYGB rats exhibit no desensitization to the effect of exogenous GLP-1 or its agonists, respectively, which is in consistent with previous reports [43].

Considering our findings that do not support a role of GLP-1, we have to presume that other mechanisms need to be considered in order to explain the absence of a compensatory decrease in energy expenditure after RYGB surgery in rats. One possible mechanism consists of a higher energy requirement due to the significant hypertrophic changes of the small intestine after RYGB surgery [16,37]. In fact, increased GLP-2 levels after RYGB may be responsible for mucosal hypertrophy, and this may help to limit malabsorptive consequences of the RYGB surgery [37]. GLP-2 has well-characterized, positive effects on epithelial proliferation particularly in the small

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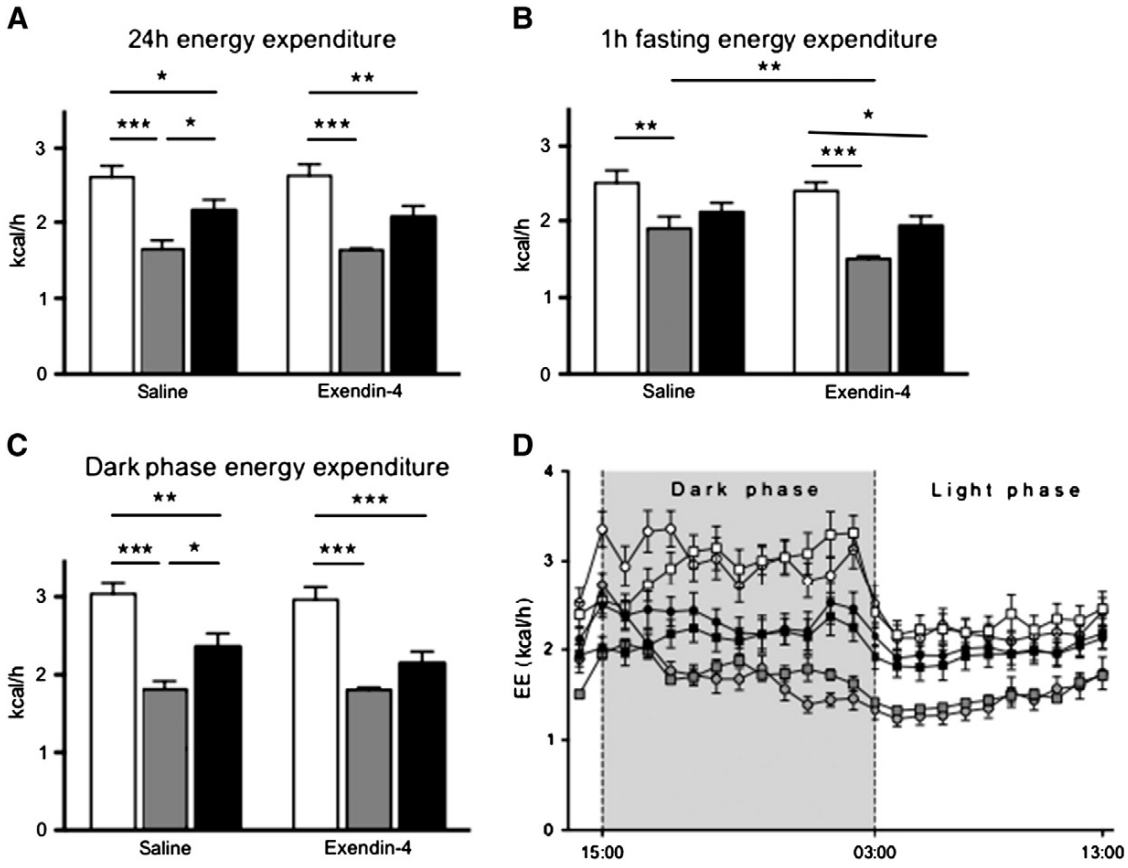

Fig. 7. A. Mean 24 h energy expenditure. B. Average energy expenditure during the first hour after Ex-4 and saline injections, respectively (1400–1500). C. Average energy expenditure during the dark phase. D. Time course of 24 h energy expenditure after Exendin-4 (5 μg/kg) and saline s.c. administration. In A, B and C sham-operated rats ad libitum fed are shown by white columns, sham-operated BWm rats by gray columns and RYGB rats by black columns. In D a similar color code is used with squares representing Exendin-4 and circles representing saline treated rats. As two-way ANOVA revealed a significant F ratio, a Bonferroni post hoc test was used to determine differences between groups (*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$). Data are shown as mean values ± SEM.

intestine [44] leading to an increased resorptive surface by enhanced crypt cell proliferation and reduced apoptosis of enterocytes [45,46]. Because the gastrointestinal tract is a metabolically very active organ [47], its hypertrophy is likely to contribute to the increase in energy expenditure after RYGB especially in response to a meal.

Differences in energy expenditure between RYGB and control rats might also be related to alterations in postprandial (or diet-induced) thermogenesis [16], even though they seem to be GLP-1 independent. Diet-induced thermogenesis is mediated in part by BAT [48,49], and it has recently been found that BAT in adult humans correlated negatively with BMI, fasting glucose levels [50] and non-alcoholic fatty liver disease [51]. However, using 18F-FDG PET/CT imaging for measurement of the metabolic activity of brown adipose tissue in RYGB and sham-operated rats, Hankir et al. were unable to demonstrate an increase in BAT activity, suggesting that other mechanisms are involved to explain the increased energy expenditure after RYGB [52]. Furthermore, there was no difference in the UCP-1 mRNA content of BAT between the two groups [52].

Our study has several limitations. First, we decided to study acute effects of GLP-1 receptor modulation on energy expenditure after RYGB surgery without investigating chronic effects. This experimental approach was based on previous results demonstrating that our indirect calorimetry system allowed us to specifically detect short-term changes in energy expenditure [29,30]. However, there is some evidence that the acute administration of GLP-1 agonists decreases energy expenditure, potentially as a direct consequence of the reduction in food intake [53]. Consistently, Ex-4 treatment led to a decrease in fasting energy expenditure in our study, which was

Table 2
RM two-way ANOVA values for 24 hour energy expenditure as well as for energy expenditure, food intake, physical activity and respiratory quotient during the dark phase after Ex-4 and saline treatment, respectively, as a function of surgical group treatment.

	Surgical group	Treatment group	Interaction
24 h energy expenditure	$F(2,22) = 17.8$, $p < 0.001$	$F(1,21) = 0.15$, $p = 0.69$	$F(2,22) = 0.19$, $p = 0.82$
1 h energy expenditure (1400–1500, no food)	$F(2,22) = 9.8$, $p < 0.001$	$F(1,22) = 15.6$, $p < 0.001$	$F(2,22) = 1.93$, $p = 0.17$
Energy expenditure (dark phase, with food)	$F(2,22) = 22.02$, $p < 0.001$	$F(1,22) = 2.1$, $p = 0.16$	$F(2,22) = 0.75$, $p = 0.48$
Food intake (dark phase)	$F(2,21) = 29.70$, $p < 0.001$	$F(1,21) = 52.73$, $p < 0.001$	$F(2,21) = 16.92$, $p < 0.001$
Physical activity (dark phase, with food)	$F(2,22) = 1.24$, $p = 0.31$	$F(1,22) = 7.66$, $p = 0.011$	$F(2,22) = 1.16$, $p = 0.33$
1 h physical activity (1400–1500, no food)	$F(2,22) = 2.84$, $p = 0.08$	$F(1,22) = 12.80$, $p < 0.01$	$F(2,22) = 1.31$, $p = 0.29$
Respiratory quotient (dark phase, with food)	$F(2,22) = 0.37$, $p = 0.695$	$F(1,22) = 4.88$, $p = 0.038$	$F(2,22) = 1.05$, $p = 0.37$

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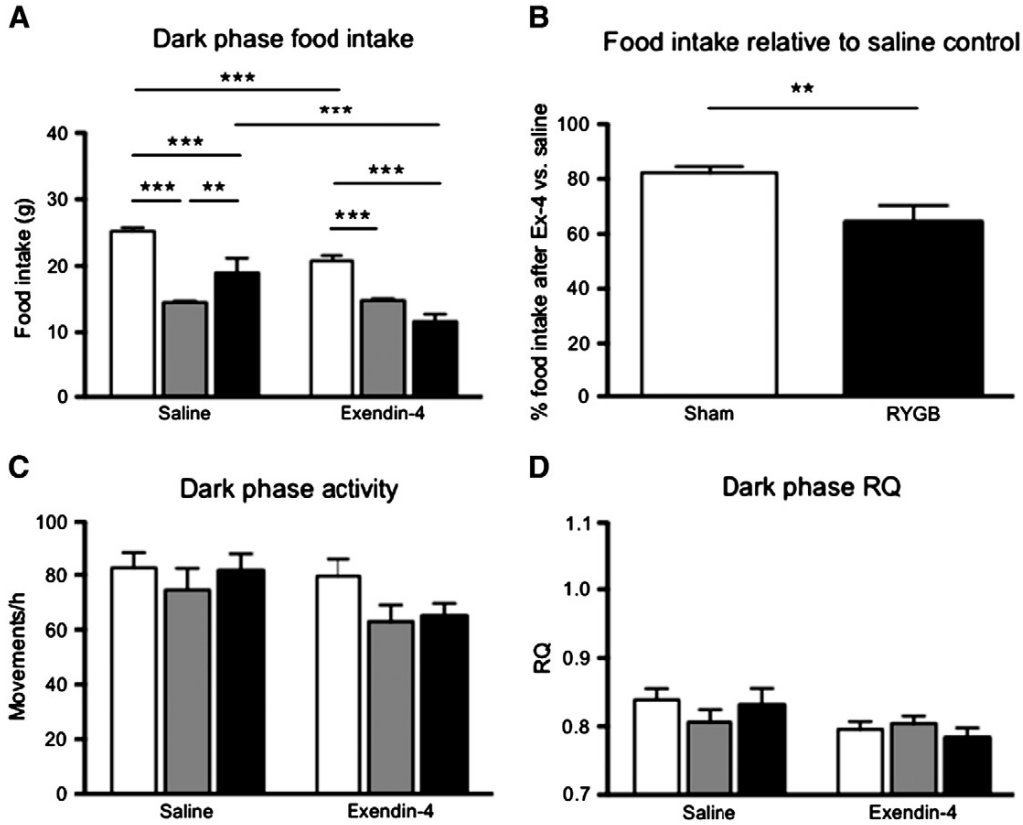

Fig. 8. A. Average spontaneous food intake during the dark phase. B. Percentage change in dark phase food intake after Ex-4 treatment compared to saline treatment. C. Average physical activity during the dark phase. D. Average Respiratory Quotient during the dark phase after Exendin-4 (5 μg/kg) and saline s.c. administration for sham-operated rats ad libitum fed (white columns), sham-operated BWm rats (gray columns) and RYGB rats (black columns). As two-way ANOVA revealed a significant F ratio, a Bonferroni post hoc test was used to determine differences between groups (*** = $p < 0.001$, ** = $p < 0.01$). Data are shown as mean values ± SEM.

associated with reduced physical activity probably due to reduced food-seeking behavior. Because the acute effects of a single-dose injection may be very different from the effects of chronic alterations in hormone signaling as observed after RYGB surgery, it would be important to see whether chronic GLP-1 receptor agonism or antagonism does have an effect on energy expenditure after RYGB or not, e.g. by using subcutaneous mini-pump systems. It would also be interesting to compare the effects of peripheral and central GLP-1 receptor modulation after RYGB surgery. Second, we only performed single dose studies. Since the mechanisms underlying the control of energy expenditure and food intake, respectively, by GLP-1 may not be identical, the absence of an acute effect of the respective compounds on energy expenditure could still be due to an ineffective dose, even though we observed the expected effects on food intake. However, the decrease in fasting energy expenditure after Ex-4 injection suggests that the acute administration of a higher dose is unlikely to yield results that would support our hypothesis, i.e. an effect consistent with the idea that elevated GLP-1 post RYGB contributes to the increase in energy expenditure. Third, endogenous GLP-1 levels were not routinely measured in our RYGB rat model. However, we have previously demonstrated increased postprandial GLP-1 levels in this model on several occasions [14,43].

In summary, our data suggest that acute modulation of GLP-1 signaling is not directly involved in the altered energy expenditure after RYGB surgery in rats. The underlying mechanisms leading to the changes in energy expenditure in RYGB rats therefore remain unclear. It seems likely that altered energy expenditure is caused by the combined effects of several different factors rather than by just one specific gut hormone.

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References

[1] Obesity: preventing and managing the global epidemic. World Health Organ Tech. Rep. Ser 2000;894:i–xii, 1–253.
[2] Adams TD, Davidson LE, Litwin SE, Kolotkin RL, Lamonte MJ, Pendleton RC, et al. Health benefits of gastric bypass surgery after 6 years. JAMA 2012;308:1122–31.
[3] Carlsson LM, Peltonen M, Ahlin S, Anveden A, Bouchard C, Carlsson B, et al. Bariatric surgery and prevention of type 2 diabetes in Swedish obese subjects. N Engl J Med 2012;367:695–704.
[4] Mingrone G, Panunzi S, De GA, Guidone C, Iaconelli A, Leccesi L, et al. Bariatric surgery versus conventional medical therapy for type 2 diabetes. N Engl J Med 2012;366:1577–85.
[5] Schauer PR, Kashyap SR, Wolski K, Brethauer SA, Kirwan JP, Pothier CE, et al. Bariatric surgery versus intensive medical therapy in obese patients with diabetes. N Engl J Med 2012;366:1567–76.
[6] Buchwald H, Oien DM. Metabolic/bariatric surgery Worldwide 2008. Obes Surg 2009;19:1605–11.
[7] Korner J, Bessler M, Cirilo LJ, Conwell IM, Daud A, Restuccia NL, et al. Effects of Roux-en-Y gastric bypass surgery on fasting and postprandial concentrations of plasma ghrelin, peptide YY, and insulin. J Clin Endocrinol Metab 2005;90:359–65.
[8] le Roux CW, Aylwin SJ, Batterham RL, Borg CM, Coyle F, Prasad V, et al. Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. Ann Surg 2006;243:108–14.

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[9] le Roux CW, Welbourn R, Werling M, Osborne A, Kokkinos A, Laurenius A, et al. Gut hormones as mediators of appetite and weight loss after Roux-en-Y gastric bypass. Ann Surg 2007;246:780–5.
[10] Laurenius A, Larsson I, Bueter M, Melanson KJ, Bosaeus I, Forslund HB, et al. Changes in eating behaviour and meal pattern following Roux-en-Y gastric bypass. Int J Obes (Lond) 2012;36:348–55.
[11] le Roux CW, Bueter M, Theis N, Werling M, Ashrafian H, Lowenstein C, et al. Gastric bypass reduces fat intake and preference. Am J Physiol Regul Integr Comp Physiol 2011;301:R1057–66.
[12] Shin AC, Zheng H, Pistell PJ, Berthoud HR. Roux-en-Y gastric bypass surgery changes food reward in rats. Int J Obes (Lond) 2011;35:642–51.
[13] Zheng H, Shin AC, Lenard NR, Townsend RL, Patterson LM, Sigalet DL, Berthoud HR. Meal patterns, satiety, and food choice in a rat model of Roux-en-Y gastric bypass surgery. Am J Physiol Regul Integr Comp Physiol 2009;297:R1273–82.
[14] Bueter M, Miras AD, Chichger H, Fenske W, Ghatei MA, Bloom SR, et al. Alterations of sucrose preference after Roux-en-Y gastric bypass. Physiol Behav 2011;104:709–21.
[15] Hajnal A, Kovacs P, Ahmed T, Meirelles K, Lynch CJ, Cooney RN. Gastric bypass surgery alters behavioral and neural taste functions for sweet taste in obese rats. Am J Physiol Gastrointest Liver Physiol 2010;299:G967–79.
[16] Bueter M, Lowenstein C, Olbers T, Wang M, Cluny NL, Bloom SR, et al. Gastric bypass increases energy expenditure in rats. Gastroenterology 2010;138:1845–53.
[17] Stylopoulos N, Hoppin AG, Kaplan LM. Roux-en-Y gastric bypass enhances energy expenditure and extends lifespan in diet-induced obese rats. Obesity (Silver Spring) 2009;17:1839–47.
[18] Wadden TA, Foster GD, Letizia KA, Mullen JL. Long-term effects of dieting on resting metabolic rate in obese outpatients. JAMA 1990;264:707–11.
[19] Shin AC, Zheng H, Townsend RL, Sigalet DL, Berthoud HR. Meal-induced hormone responses in a rat model of Roux-en-Y gastric bypass surgery. Endocrinology 2010;151:1588–97.
[20] Rubino F, Gagner M, Gentileschi P, Kini S, Fukuyama S, Feng J, et al. The early effect of the Roux-en-Y gastric bypass on hormones involved in body weight regulation and glucose metabolism. Ann Surg 2004;240:236–42.
[21] Abbott CR, Monteiro M, Small CJ, Sajedi A, Smith KL, Parkinson JR, et al. The inhibitory effects of peripheral administration of peptide YY(3–36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal–brainstem–hypothalamic pathway. Brain Res 2005;1044:127–31.
[22] Chelikani PK, Haver AC, Reidelberger RD. Intravenous infusion of peptide YY(3–36) potently inhibits food intake in rats. Endocrinology 2005;146:879–88.
[23] Kinzig KP, D'Alessio DA, Seeley RJ. The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. J Neurosci 2002;22:10470–6.
[24] Schick RR, Zimmermann JP, vorm WT, Schusdziarra V. Peptides that regulate food intake: glucagon-like peptide 1-(7-36) amide acts at lateral and medial hypothalamic sites to suppress feeding in rats. Am J Physiol Regul Integr Comp Physiol 2003;284:R1427–35.
[25] Williams DL, Baskin DG, Schwartz MW. Evidence that intestinal glucagon-like peptide-1 plays a physiological role in satiety. Endocrinology 2009;150:1680–7.
[26] Osaka T, Endo M, Yamakawa M, Inoue S. Energy expenditure by intravenous administration of glucagon-like peptide-1 mediated by the lower brainstem and sympathoadrenal system. Peptides 2005;26:1623–31.
[27] Conarello SL, Li Z, Ronan J, Roy RS, Zhu L, Jiang G, et al. Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin resistance. Proc Natl Acad Sci U S A 2003;100:6825–30.
[28] Bueter M, Abegg K, Seyfried F, Lutz TA, le Roux CW. Roux-en-Y gastric bypass operation in rats. J Vis Exp 2012:e3940.
[29] Wielinga PY, Alder B, Lutz TA. The acute effect of amylin and salmon calcitonin on energy expenditure. Physiol Behav 2007;91:212–7.
[30] Wielinga PY, Lowenstein C, Muff S, Munz M, Woods SC, Lutz TA. Central amylin acts as an adiposity signal to control body weight and energy expenditure. Physiol Behav 2010;101:45–52.
[31] Nestoridi E, Kvas S, Kucharczyk J, Stylopoulos N. Resting energy expenditure and energetic cost of feeding are augmented after Roux-en-Y gastric bypass in obese mice. Endocrinology 2012;153:2234–44.
[32] Saeidi N, Nestoridi E, Kucharczyk J, Uygun MK, Yarmush ML, Stylopoulos N. Sleeve gastrectomy and Roux-en-Y gastric bypass exhibit differential effects on food preferences, nutrient absorption and energy expenditure in obese rats. Int J Obes (Lond) 2012;36:1396–402.
[33] Chelikani PK, Haver AC, Reidelberger RD. Intravenous infusion of glucagon-like peptide-1 potently inhibits food intake, sham feeding, and gastric emptying in rats. Am J Physiol Regul Integr Comp Physiol 2005;288:R1695–706.
[34] Lockie SH, Heppner KM, Chaudhary N, Chabenne JR, Morgan DA, Veyrat-Durebex C, et al. Direct control of brown adipose tissue thermogenesis by central nervous system glucagon-like peptide-1 receptor signaling. Diabetes 2012;61:2753–62.
[35] Ashrafian H, le Roux CW. Metabolic surgery and gut hormones—a review of bariatric entero-humoral modulation. Physiol Behav 2009;97:620–31.
[36] Laferrere B, Swerdlow N, Bawa B, Arias S, Bose M, Olivan B, et al. Rise of oxyntomodulin in response to oral glucose after gastric bypass surgery in patients with type 2 diabetes. J Clin Endocrinol Metab 2010;95:4072–6.
[37] le Roux CW, Borg C, Wallis K, Vincent RP, Bueter M, Goodlad R, et al. Gut hypertrophy after gastric bypass is associated with increased glucagon-like peptide 2 and intestinal crypt cell proliferation. Ann Surg 2010;252:50–6.
[38] Adams SH, Lei C, Jodka CM, Nikoulina SE, Hoyt JA, Gedulin B, et al. PYY[3–36] administration decreases the respiratory quotient and reduces adiposity in diet-induced obese mice. J Nutr 2006;136:195–201.
[39] Dakin CL, Small CJ, Park AJ, Seth A, Ghatei MA, Bloom SR. Repeated ICV administration of oxyntomodulin causes a greater reduction in body weight gain than in pair-fed rats. Am J Physiol Endocrinol Metab 2002;283:E1173–7.
[40] Wynne K, Park AJ, Small CJ, Patterson M, Ellis SM, Murphy KG, et al. Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. Diabetes 2005;54:2390–5.
[41] Liu Y, Zhou Y, Wang Y, Geng D, Liu J. Roux-en-Y gastric bypass-induced improvement of glucose tolerance and insulin resistance in type 2 diabetic rats are mediated by glucagon-like peptide-1. Obes Surg 2011;21:1424–31.
[42] Taqi E, Wallace LE, de Heuvel E, Chelikani PK, Zheng H, Berthoud HR, et al. The influence of nutrients, biliary-pancreatic secretions, and systemic trophic hormones on intestinal adaptation in a Roux-en-Y bypass model. J Pediatr Surg 2010;45:987–95.
[43] Fenske WK, Bueter M, Miras AD, Ghatei MA, Bloom SR, le Roux CW. Exogenous peptide YY3–36 and Exendin-4 further decrease food intake, whereas octreotide increases food intake in rats after Roux-en-Y gastric bypass. Int J Obes (Lond) 2012;36:379–84.
[44] Drucker DJ. Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis. Mol Endocrinol 2003;17:161–71.
[45] Tsai CH, Hill M, Drucker DJ. Biological determinants of intestinotrophic properties of GLP-2 in vivo. Am J Physiol 1997;272:G662–8.
[46] Tsai CH, Hill M, Asa SL, Brubaker PL, Drucker DJ. Intestinal growth-promoting properties of glucagon-like peptide-2 in mice. Am J Physiol 1997;273:E77–84.
[47] Cant JP, McBride BW, Croom Jr WJ. The regulation of intestinal metabolism and its impact on whole animal energetics. J Anim Sci 1996;74:2541–53.
[48] Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev 2004;84:277–359.
[49] Rothwell NJ, Stock MJ. A role for brown adipose tissue in diet-induced thermogenesis. Obes Res 1997;5:650–6.
[50] Lee P, Greenfield JR, Ho KK, Fulham MJ. A critical appraisal of the prevalence and metabolic significance of brown adipose tissue in adult humans. Am J Physiol Endocrinol Metab 2010;299:E601–6.
[51] Yilmaz Y, Ones T, Purnak T, Ozguven S, Kurt R, Atug O, et al. Association between the presence of brown adipose tissue and non-alcoholic fatty liver disease in adult humans. Aliment Pharmacol Ther 2011;34:318–23.
[52] Hankir M, Bueter M, Gsell W, Seyfried F, Khalil M, Smith KL, et al. Increased energy expenditure in gastric bypass rats is not caused by activated brown adipose tissue. Obes Facts 2012;5:349–58.
[53] Baraboi ED, Smith P, Ferguson AV, Richard D. Lesions of area postrema and subfornical organ alter exendin-4-induced brain activation without preventing the hypophagic effect of the GLP-1 receptor agonist. Am J Physiol Regul Integr Comp Physiol 2010;298:R1098–110.

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7. Unpublished results

As previously mentioned (see 3.1.2), there are currently two major issues that need to be considered when analyzing EE data. One is the influence of potential differences in body weight and body composition on EE. The other is the importance of performing experiments at thermoneutrality, because performing them at lower temperatures can either cause thermogenesis-induced artifacts that are interpreted as "real" effects, or it can mask small differences in EE between groups due to an overall thermogenesis-induced increase in EE. Since these questions repeatedly came up when presenting our findings of altered EE in RYGB rats, we wanted to address both of them.

7.1. Changes in whole body composition after RYGB surgery

7.1.1. Background

When alterations in EE in our RYGB rat model were first reported, measurement of body composition using a rodent computerized tomography (CT) scanner was included in the study.[124] Based on previous careful validation of scanner and software,[248] the region between lumbar vertebrae L1 and L5 was scanned to estimate whole body composition since a strong correlation between L1-L5 and whole body measurement had previously been shown. This analysis revealed a higher lean mass in RYGB compared to sham operated body weight-matched (BWM) rats (80.9 ± 2.4 g in RYGB vs 71.0 ± 1.1 g in BWM); however, the measured L1-L5 adipose tissue mass was also higher (11.6 ± 1.3 g in RYGB vs 5.3 ± 0.9 g in BWM). All factors combined this caused a difference of over 15 g in total measured L1-L5 tissue mass between RYGB and BWM rats even though there was no difference in body weight (408.7 ± 9.4 g in RYGB vs 412.2 ± 3.0 g in BWM).[124] This suggests that, although verified for unoperated rats, L1-L5 analysis may not be representative of whole body composition in RYGB rats. We therefore wanted to evaluate whole body composition, i.e. lean mass, subcutaneous fat mass and visceral fat mass, in RYGB, BWM and sham operated ad libitum fed (AL) rats. We further wanted to assess the correlation of the obtained whole body results with results obtained by only analyzing the lumbar region in the same scans for each group.

7.1.2. Design

The same whole body CT images that were used for bone density analysis 14 weeks after surgery (see 5) were analyzed for lean mass, subcutaneous fat mass and visceral fat mass with the validated Aloka software.[248] Body weight was stable in all groups at this time point and was significantly higher in AL than in RYGB and BWM rats ($p < .001$, Figure 2)

Before calculating lean and fat mass, manual image-by-image correction of software miscategorization was performed. This included (1) correct definiton of subcutaneous fat that had been miscategorized as visceral fat and vice versa, (2) exclusion of lung tissue from the analysis since this was often miscatecorized as visceral fat and (3) exclusion of intestinal content containing air pockets that were often miscategorized as visceral fat (Figure 3). Calculations of whole body lean and fat mass and L1-L6 lean and fat mass were then performed on the same set of corrected images for each animal (Figure 4). We decided to use L1-L6 since this had been shown to have a slightly higher accuracy of whole body estimation than L1-L5 in normal rats.[248]

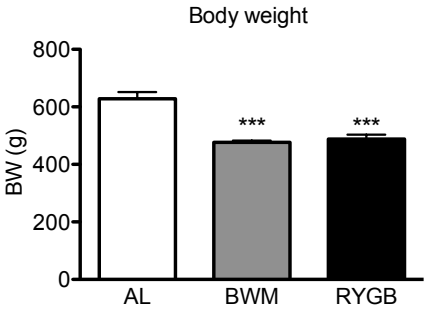

Figure 2 Body weight of sham operated ad libitum fed (AL, n = 9), sham operated body weight-matched (BWM, n = 8) and gastric bypass operated (RYGB, n = 12) rats 14 weeks after surgery. ***p < .001 vs AL. Data represent mean ± SEM.

Data were analyzed by one-way ANOVA followed by Bonferroni post-hoc testing to find differences between surgery groups. Furthermore, one-way ANOVA followed by Bonferroni post-hoc testing was used to analyze the effect of ambient temperature within the individual surgery groups. Linear regression analyses were performed to determine the correlation between whole body and L1-L6 measurents for each surgery group separately. Data are represented as mean ± SEM and statistical significance was established at $p < .05$.

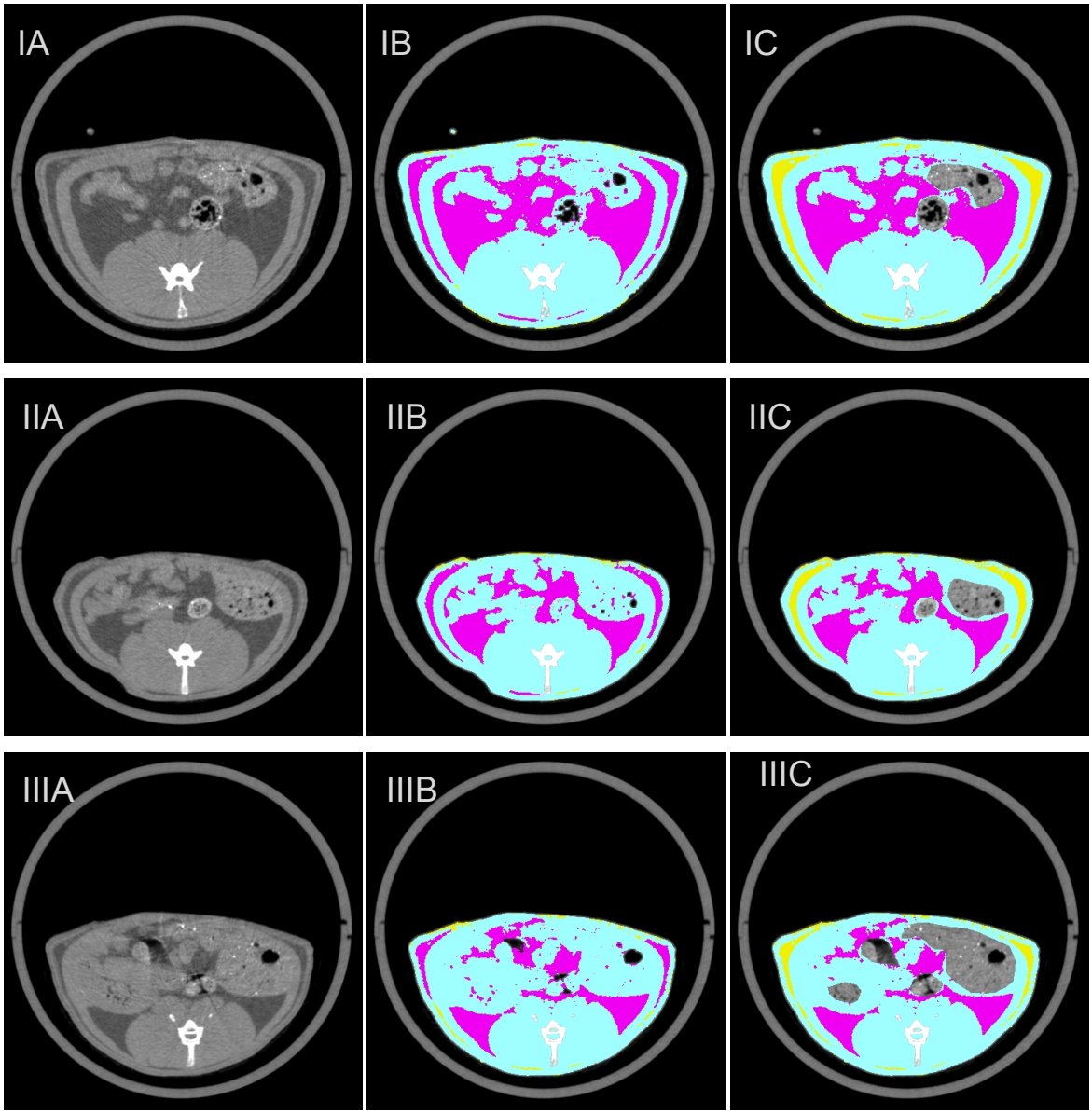

Figure 3 Representative images of AL (I), BWM (II) and RYGB (III) rats in gray scale (A), after automated lean and fat mass categorization (B) and after manual correction of lean and fat mass categorization (C). Blue = lean mass; magenta = visceral fat mass; yellow = subcutaneous fat mass.

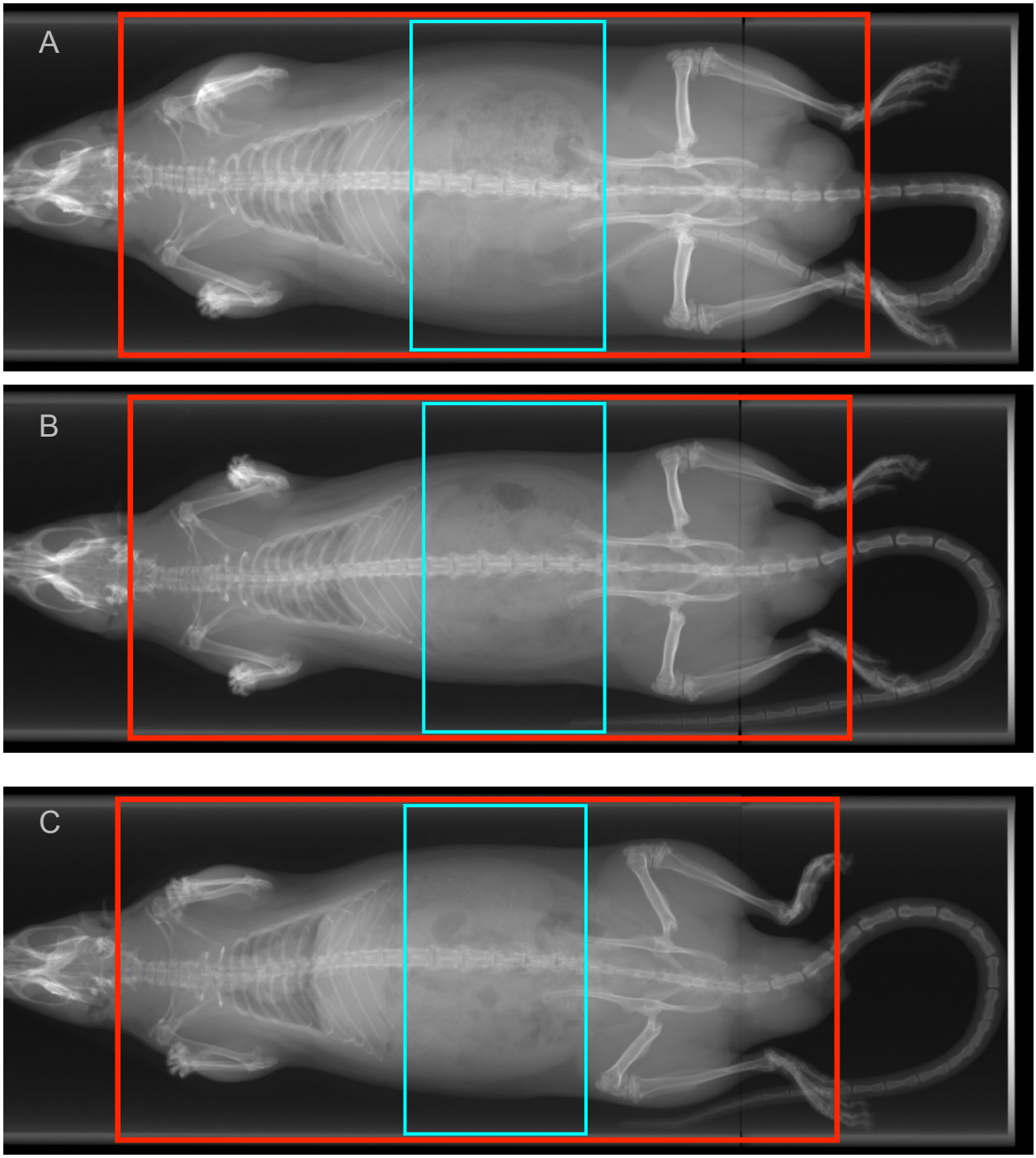

Figure 4 Representative scout images of AL (A), BWM (B) and RYGB (C) rats. Red frame represents region used for calculation of whole body composition, blue frame represents region used for calculation of L1-L6 body composition.

7.1.3. Results

7.1.3.1. Body composition

Body composition as calculated by whole body and L1-L6 analysis is illustrated in Figure 5. Lean mass, visceral fat mass and subcutaneous fat mass were all significantly decreased in RYGB and BWM compared to AL rats, but there was no difference between RYGB and BWM rats (Figure 6).

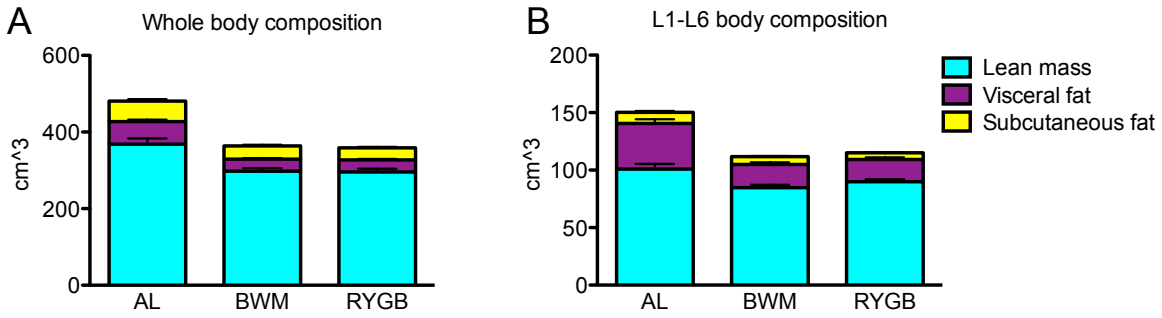

Figure 5 Estimated whole body (A) and L1-L6 (B) lean mass, visceral fat mass and subcutaneous fat mass in sham operated ad libitum fed (AL, n = 9), sham operated body weight-matched (BWM, n = 8) and gastric bypass operated (RYGB, n = 12) rats 14 weeks after surgery. Data represent mean ± SEM.

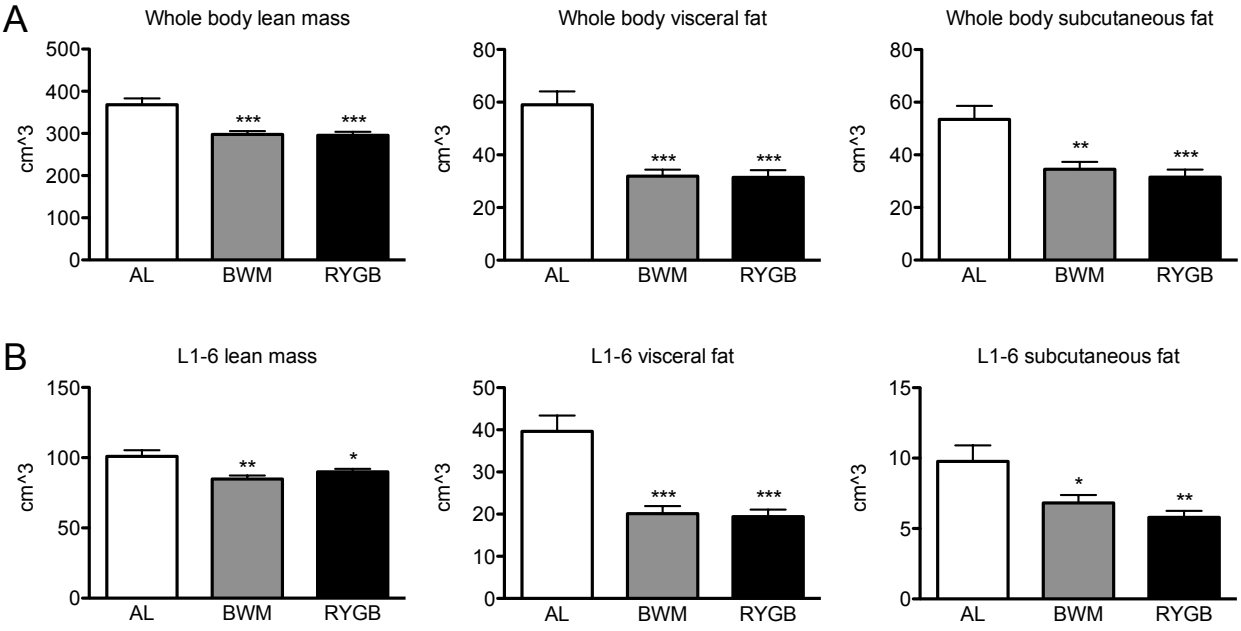

Figure 6 Estimated whole body (A) and L1-L6 (B) lean mass, visceral fat mass and subcutaneous fat mass in sham operated ad libitum fed (AL, n = 9), sham operated body weight-matched (BWM, n = 8) and gastric bypass operated (RYGB, n = 12) rats 14 weeks after surgery. *p < .05, **p < .01, ***p < .001 vs AL. Data represent mean ± SEM.

7.1.3.2. Correlation between whole body and L1-L6 measurements

For all surgery groups, slopes of the linear regression analyses of whole body compared to L1-L6 lean mass, visceral fat mass and subcutaneous fat mass differed significantly from zero, suggesting a strong relationship between the two measurements (Figure 7 and Table 1). However, there was a significant difference between AL and RYGB rats in the slope of visceral fat mass correlation and between all groups in the y-intercept of lean mass correlation. Furthermore, while the high R^2 values of visceral fat mass correlation suggest a very well fitted regression line for all surgery groups, the R^2 values of lean mass and subcutaneous fat mass correlations were much lower in RYGB rats (Table 1).

Table 1 Linear regression analysis of calculated tissue mass based on either whole body or L1-L6 measurement. S.E.E. standard error of estimate. *p < .05, **p< .01, ***p < .001 vs AL; §p < .05 vs BWM; ++p < 0.01, +++p < .001 vs zero slope. Data represent mean ± SEM.

	AL	BWM	RYGB
Lean mass			
Whole body	368.1 ± 15.0	297.5 ± 8.0***	295.5 ± 8.6***
L1-L6	100.90 ± 4.47	84.79 ± 2.54**	89.93 ± 2.18*
Slope	3.191 ± 0.391^{+++}	3.113 ± 0.227^{+++}	2.984 ± 0.816^{++}
Y-intercept	46.09 ± 39.73	33.59 ± 19.28*	27.22 ± 73.59***§
R^2	0.9050	0.9692	0.5723
S.E.E.	14.81	4.313	20.41
Visceral fat			
Whole body	59.05 ± 5.07	31.93 ± 2.48***	31.48 ± 2.72***
L1-L6	39.65 ± 3.73	20.13 ± 1.80***	19.40 ± 1.70***
Slope	1.357 ± 0.040^{+++}	1.370 ± 0.066^{+++}	1.585 ± 0.075*$^{+++}$
Y-intercept	5.236 ± 1.635	4.353 ± 1.367	0.744 ± 1.512
R^2	0.9940	0.9862	0.9782
S.E.E.	1.259	0.8907	1.458
Subcutaneous fat			
Whole body	53.49 ± 5.15	34.53 ± 2.80**	31.49 ± 2.93***
L1-L6	9.774 ± 1.128	6.823 ± 0.557*	5.791 ± 0.467**
Slope	4.228 ± 0.646^{+++}	4.355 ± 1.026^{++}	4.439 ± 1.397^{++}
Y-intercept	12.17 ± 0.65	4.815 ± 7.16	5.792 ± 8.38
R^2	0.8594	0.7501	0.5023
S.E.E.	6.188	4.279	7.499

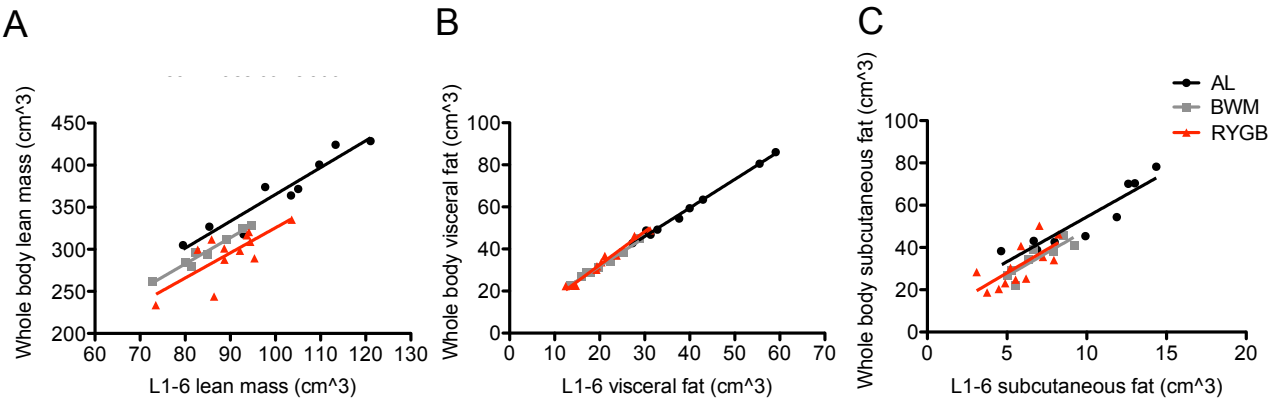

Figure 7 Relationships of lean mass (A), visceral fat mass (B) and subcutaneous fat mass (C) calculated based on either whole body or L1-L6 measurement in sham operated ad libitum fed (AL, n = 9), sham operated body weight-matched (BWM, n = 8) and gastric bypass operated (RYGB, n = 12) rats 14 weeks after surgery.

7.1.4. Conclusions

Our data show that 3 months after surgery, when body weight was stable (Figure 2), there was no difference in body composition or in total lean mass between RYGB and BWM rats. This suggests that the increase in EE observed after RYGB-induced weight loss compared to weight loss achieved by food restriction[124,249] is not due to smaller loss of lean tissue. However, since lean mass is very heterogenous and consists of various tissues with very different metabolic requirements (see 3.1.2), we cannot completely exclude that differences in lean mass composition account for the observed alterations. In fact, it has been shown that RYGB rats display an almost two-fold increase in gut weight compared to sham operated rats,[124,250] which, if associated with a decrease in a metabolically less active lean tissue, could cause a relative increase in total lean mass EE.

We also confirmed that generally there is a very good correlation between body composition measured in abdominal L1-L6 or whole body CT scans. However, linear regression analysis revealed that this correlation is not as accurate in RYGB rats as it is in AL and BWM rats. First, the slope of the regression line for visceral fat was significantly higher in RYGB than in AL rats, which means that in case of high levels of visceral adiposity, L1-L6 analysis may underestimate whole body visceral fat in RYGB rats, while in case of very low visceral adiposity, L1-L6 analysis may overestimate whole body adiposity in RYGB compared to AL rats. Second, the y-intercept of the regression line for lean mass was significantly lower in BWM compared to AL rats and in RYGB compared to AL and BWM rats. This means that L1-L6 analysis may generally overestimate whole body lean mass in BWM and even more in RYGB rats. Third, while the data fitted the regression line very well in AL and BWM rats, this was not the case for lean mass and subcutaneous fat in RYGB rats, suggesting that L1-L6 analysis is not as accurate for estimation of whole body lean mass and subcutaneous fat in RYGB as in sham rats. Together, these results show that within the examined body weight range, L1-L6 analysis did not significantly change the outcome of body

composition estimation compared to whole body analysis. However, the linear regression analyses suggest that whenever possible, whole body CT scans should be performed since it results in a more accurate estimation of body composition in RYGB rats. The two main limitations of this study were (1) the small group sizes and relatively small body weight range of the animals within groups and (2) the lack of EE data of the animals used for CT evaluation. Ideally, effects of RYGB surgery on body composition should be evaluated in a large group of animals including diet-induced obese rats and correlated with EE recordings in the same animals.

7.2. Determination of the thermoneutral zone of RYGB and sham rats

7.2.1. Background

We and others have repeatedly shown that the decrease in EE that occurs as a compensatory response to weight loss (e.g. induced by food restriction) is attenuated by RYGB surgery.[124,125,249] However, as previously discussed, the ambient temperature at which EE is recorded can have a strong influence on the outcome of such measurements;[70,71] and the aforementioned studies were performed at room temperature, i.e. at around 22°C. Since there is some evidence of an influence of hormonal signaling on the TNZ,[251] we hypothesized that changes in hormone levels and neuroendocrine signaling and the lower fat mass after RYGB surgery could lead to a shift in the TNZ of rats to higher ambient temperatures, which would mean that the observed increase in EE was at least partly caused by higher adaptive thermogenesis. The aim of this experiment was to identify the TNZ, i.e. the LCT and the UCT, of sham operated ad libitum fed (AL), sham operated body weight-matched (BWM) and gastric bypass operated (RYGB) rats to determine whether the reported alterations in EE are partly an artifact due to differences in adaptive thermogenesis.

7.2.2. Design

16 male Wistar rats (400 - 450 g) were maintained on a normal chow diet (Provimi Kliba, Kaiseraugst, Switzerland) for 2 weeks before randomization for sham (n = 11) or RYGB surgery (n = 5) as previously described. Directly after the RYGB or sham procedure, telemetry sensors (F40-TT; Data Sciences International, St. Paul, USA) were implanted intraperitoneally for the measurement of body core temperature and physical activity. After surgery, the sham rats were further divided into an ad libitum fed (AL, n = 6) and a body weight-matched (BWM, n = 5) group. The BWM rats were food restricted to the extent that was necessary for them to maintain the same body weight as RYGB rats. Four weeks after surgery when body weight in RYGB rats had stabilized, rats were placed in an open circuit indirect calorimetry system (TSE Systems, Bad Homburg, Germany) allowing simultaneous recording of V_{O2}, V_{CO2}, and food and water intake located in an environmental chamber that allowed precise manipulation of ambient temperatures. EE, intraperitoneal temperature, physical activity, and food and water intake were recorded

throughout the measurement period. After 3 days of adaptation to the cages at 24°C, ambient temperature was increased to 32°C and maintained for a day. Temperature was then stepwise decreased by 2°C per day until a temperature of 22°C was reached. EE was calculated from V_{O2} and V_{CO2} according to the Weir formula.[252]

Data were analyzed by two-way ANOVA to detect main effects of surgery group or ambient temperature, followed by Bonferroni post-hoc testing to find differences between groups at the individual ambient temperatures. Furthermore, one-way ANOVA followed by Bonferroni post-hoc testing was used to analyze the effect of ambient temperature within the individual surgery groups. Pearson correlation coefficient was used to measure the linear correlation between body core temperature and ambient temperature. Data are represented as mean ± SEM and statistical significance was established at $p < .05$.

7.2.3. Results

7.2.3.1. Body weight

Body weight in RYGB rats decreased rapidly after surgery and was significantly lower compared to AL rats from postsurgical week 1 ($p < .01$). Weight loss in BWM rats occurred very slowly and required severe food restriction (see Figure 16); however, at the time of indirect calorimetry recordings for the determination of thermoneutrality, there was no significant difference in body weight between RYGB and BWM rats, while BW of AL rats was significantly higher ($p < .001$, Figure 8 and Figure 9). When comparing presurgical body weight with body weight at the time of indirect calorimetry recordings, two-way ANOVA revealed a significant main effect of surgery group ($F_{2,13} = 13.14$; $p < .001$) and time ($F_{1,13} = 39.24$; $p < .001$) and a significant interaction of surgery group x time ($F_{2,13} = 52.89$; $p < .001$), representing significant weight gain in AL rats and significant weight loss in RYGB and BWM rats (Figure 9).

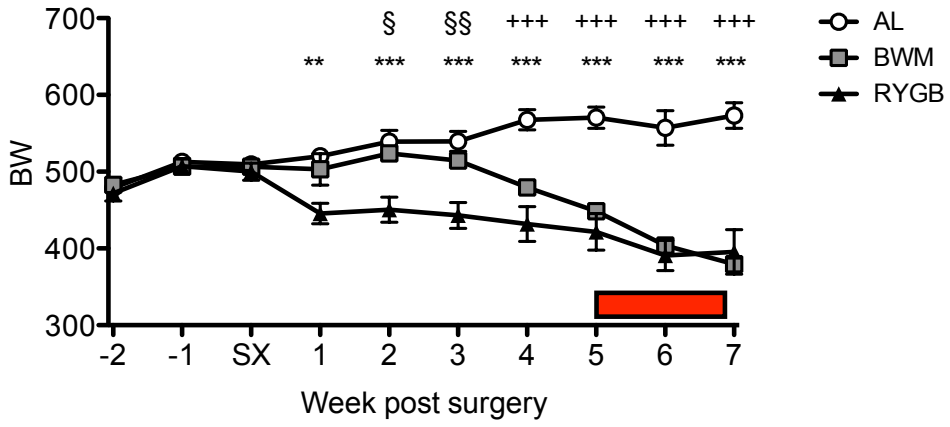

Figure 8 Body weight (BW) development 2 weeks before and 7 weeks after surgery in sham operated ad libitum fed (AL, n = 6), sham operated body weight-matched (BWM, n = 5) and gastric bypass operated (RYGB, n = 5) rats. **p < .01,

***p < .001 AL vs RYGB; +++ p < .001 AL vs BWM; §p < .05, §§p < .01 BWM vs RYGB. Data represent mean ± SEM. Red bar indicates the time of indirect calorimetry recordings for the determination of thermoneutrality.

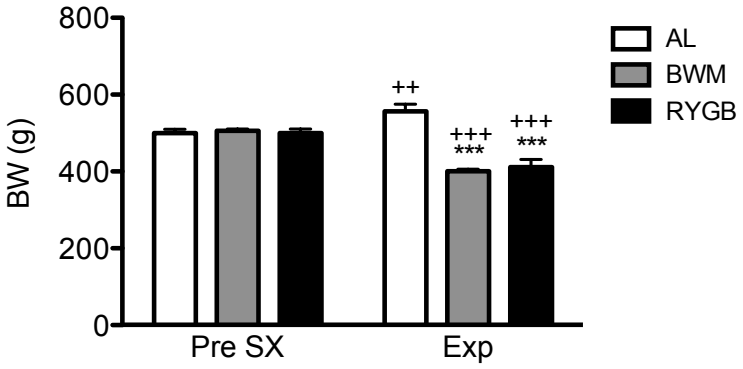

Figure 9 Body weight (BW) before surgery (Pre SX) and during indirect calorimetry recordings for the determination of thermoneutrality (Exp) in sham operated ad libitum fed (AL, n = 6), sham operated body weight-matched (BWM, n = 5) and gastric bypass operated (RYGB, n = 5) rats. ***p < .001 vs AL; ++p < .01, +++p < .001 Exp vs Pre SX. Data represent mean ± SEM.

7.2.3.2. Energy expenditure

At all measured ambient temperatures between 22 and 32°C, 24-hour EE was significantly higher in AL and RYGB compared to BWM rats (p < .001). EE was significantly higher in AL than in RYGB animals at 22 (p < .001), 24 and 32°C (p < .01), but not at ambient temperatures between 26 and 30°C (Figure 10). In AL rats, lowest EE was measured at 30°C. EE was significantly higher at 22 (p < .01), 24 (p < .05) and 32°C (p < .01, Figure 11A and Table 2). In BWM rats, lowest EE was measured at 26°C; however, the absolute values of EE were very similar between 26 and 32°C (range 6.039-6.152 kJ / h), while EE at 22°C was significantly increased (p < .001, Figure 11B and Table 2). In RYGB rats, lowest EE was measured at 28°C; however, there was no significant increase in EE compared to that value at any other ambient temperature (Figure 11C and Table 2).

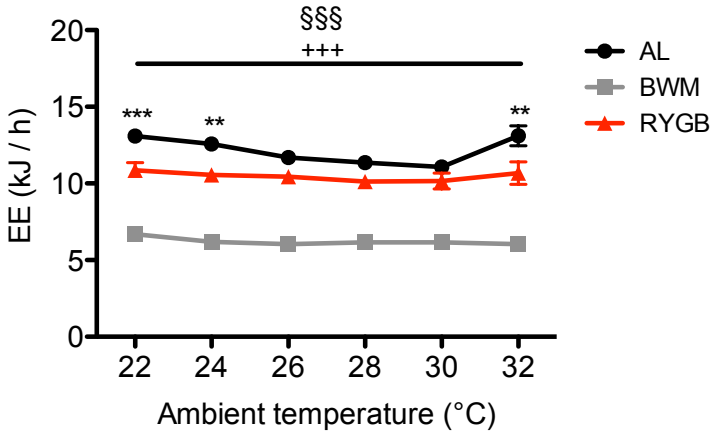

Figure 10 24-hour energy expenditure (EE) of sham operated ad libitum fed (AL, n = 6), sham operated body weight-matched (BWM, n = 5) and gastric bypass operated (RYGB, n = 5) rats at different ambient temperatures. **p < .01, ***p < .001 AL vs RYGB; +++p < .001 AL vs BWM; §§§p < .001 BWM vs RYGB. Data represent mean ± SEM.

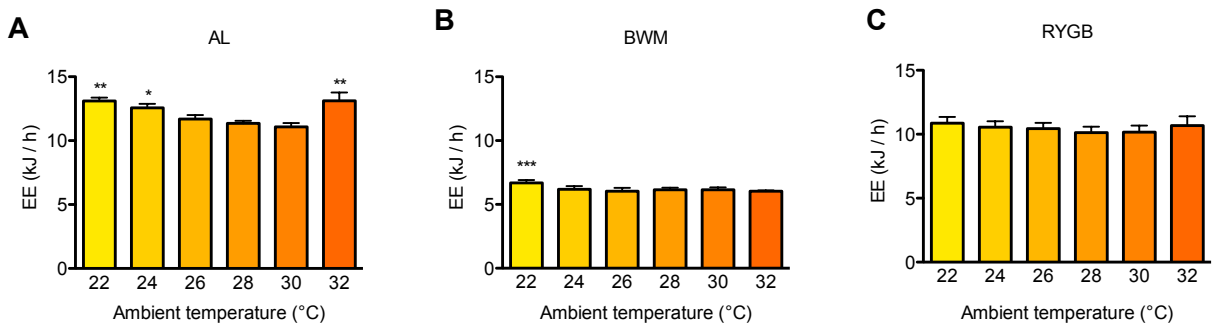

Figure 11 24-hour energy expenditure (EE) of AL (A), BWM (B) and RYGB (C) rats at different ambient temperatures. *p < .05, **p < .01, ***p < .001 vs lowest measured EE, i.e. vs EE at thermoneutrality. Data represent mean ± SEM.

Table 2 Mean values ± SEM for 24-hour energy expenditure of AL, BWM and RYGB rats at different ambient temperatures. See **Figure 10** and **Figure 11** for significances.

	22°C	24°C	26°C	28°C	30°C	32°C
AL	13.10 ± 0.26	12.57 ± 0.31	11.69 ± 0.32	11.36 ± 0.19	11.08 ± 0.31	13.11 ± 0.65
BWM	6.69 ± 0.22	6.18 ± 0.26	6.04 ± 0.26	6.15 ± 0.16	6.15 ± 0.18	6.04 ± 0.13
RYGB	10.87 ± 0.49	10.56 ± 0.46	10.44 ± 0.44	10.13 ± 0.47	10.16 ± 0.51	10.68 ± 0.73

7.2.3.3. Body core temperature

At all measured ambient temperatures between 22 and 32°C, body core temperature (T_C) was significantly higher in AL compared to BWM rats ($p < .01$). At 22 ($p < .01$), 24 ($p < .05$), 26 ($p < .01$) and 32°C ($p < .001$), T_C was also significantly higher in RYGB compared to BWM rats. There was no significant difference in T_C between AL and RYGB rats at any ambient temperature (Figure 12).

In both AL and RYGB rats, there was a significant increase in T_C at 32°C ($p < .001$ and $p < .05$, respectively, Figure 13A,C and Table 3), while there was no significant difference in T_C of BWM rats between different ambient temperatures (Figure 13B and Table 3).

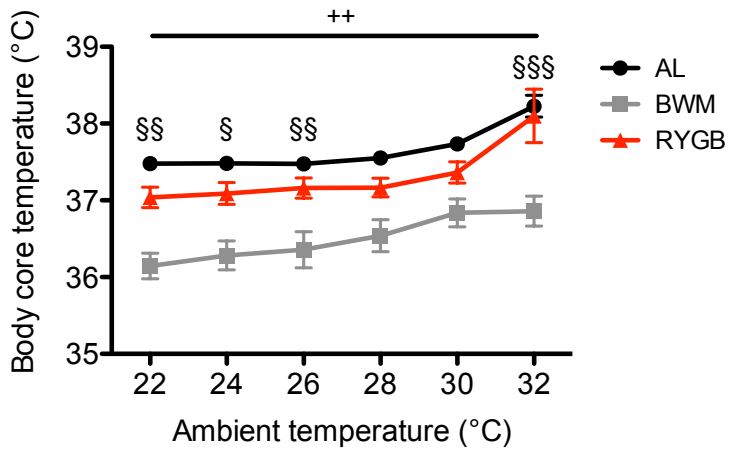

Figure 12 Body core temperature of sham operated ad libitum fed (AL, n = 6), sham operated body weight-matched (BWM, n = 5) and gastric bypass operated (RYGB, n = 5) rats at different ambient temperatures. ++p < .01 AL vs BWM; §p < .05, §§p < .01, §§§p < .001 BWM vs RYGB. Data represent mean ± SEM.

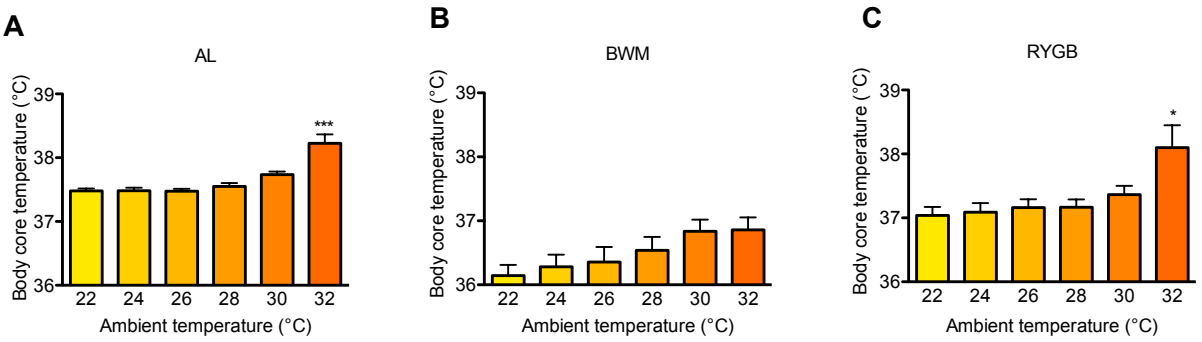

Figure 13 Body core temperature of AL (A), BWM (B) and RYGB (C) rats at different ambient temperatures. *p < .05, ***p < .001 vs all other ambient temperatures. Data represent mean ± SEM.

Table 3 Mean values ± SEM for body core temperature of AL, BWM and RYGB rats at different ambient temperatures. See **Figure 12** and **Figure 13** for significances.

	22°C	**24°C**	**26°C**	**28°C**	**30°C**	**32°C**
AL	37.48 ± 0.04	37.48 ± 0.05	37.47 ± 0.04	37.55 ± 0.05	37.73 ± 0.05	38.23 ± 0.14
BWM	36.14 ± 0.17	36.28 ± 0.19	36.36 ± 0.23	36.54 ± 0.21	36.84 ± 0.18	36.86 ± 0.20
RYGB	37.04 ± 0.13	37.09 ± 0.14	37.16 ± 0.13	37.17 ± 0.12	37.36 ± 0.14	38.10 ± 0.35

7.2.3.4. Physical activity

There was no significant difference in physical activity between groups at any measured ambient temperature between 22 and 32°C (Figure 14), although two-way ANOVA revealed a significant

main effect of surgery group ($F_{2,76}$ = 4.625; p < .05). However, physical activity seemed to be increased at 32°C in all surgery groups, although this increase only reached statistical significance in BWM rats (Figure 15 and Table 4).

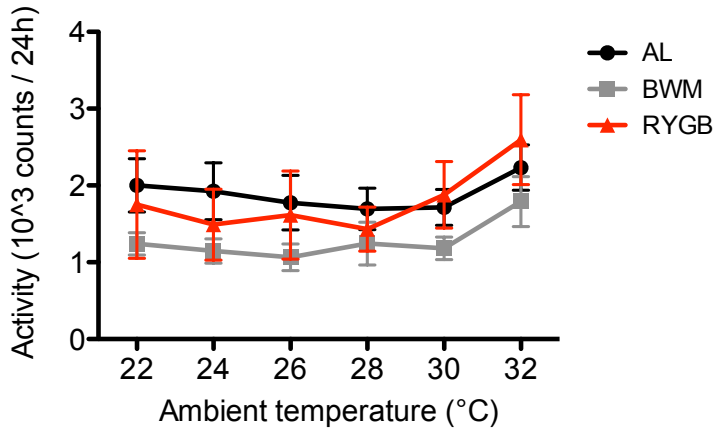

Figure 14 Physical activity of sham operated ad libitum fed (AL, n = 6), sham operated body weight-matched (BWM, n = 5) and gastric bypass operated (RYGB, n = 5) rats at different ambient temperatures. Data represent mean ± SEM.

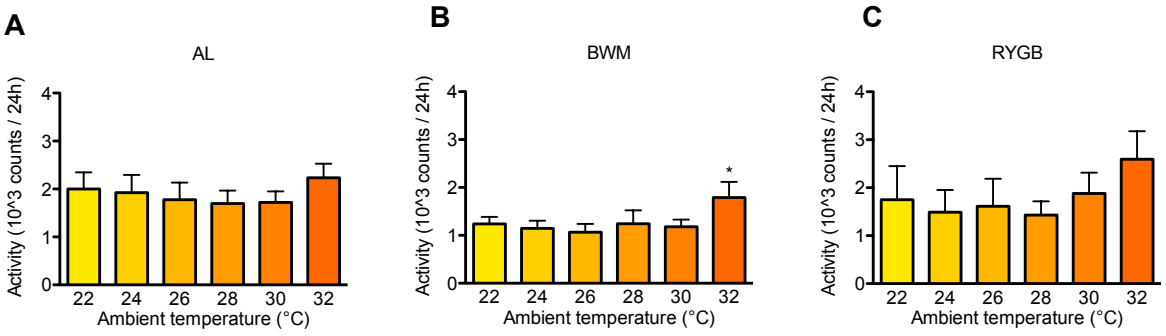

Figure 15 Physical activity of AL (A), BWM (B) and RYGB (C) rats at different ambient temperatures. *p < .05 vs all other ambient temperatures. Data represent mean ± SEM.

Table 4 Mean values ± SEM for physical activity of AL, BWM and RYGB rats at different ambient temperatures. See **Figure 14** and **Figure 15** for significances.

	22°C	24°C	26°C	28°C	30°C	32°C
AL	2002 ± 345	1924 ± 369	1777 ± 355	1696 ± 269	1716 ± 232	2232 ± 295
BWM	1240 ± 146	1148 ± 158	1067 ± 173	1244 ± 278	1181 ± 147	1791 ± 325
RYGB	1751 ± 700	1490 ± 463	1614 ± 573	1431 ± 286	1879 ± 433	2596 ± 585

7.2.3.5. Food and water intake

The amount of food required for BWM rats to maintain the same body weight as RYGB rats was significantly lower than 24-hour food intake of both AL and RYGB rats at ambient temperatures between 22 and 30°C (p < .001). Food intake of RYGB rats was only significantly decreased compared to AL rats at 24°C (p < .05, Figure 16). The difference in food intake between BWM and

the other two groups disappeared at 32°C due to a marked reduction in FI of AL and RYGB rats ($p < .01$,Figure 16, Figure 17 and Table 5).

Water intake was significantly lower in BWM compared to AL rats at ambient temperatures between 22 and 30°C ($p < .05$), but this difference disappeared at 32°C due to a reduction in water intake of AL rats. WI of RYGB rats did not significantly differ from the other two groups (Figure 18). There was no significant effect of ambient temperature on water intake within the different surgery groups (Figure 19 and Table 6).

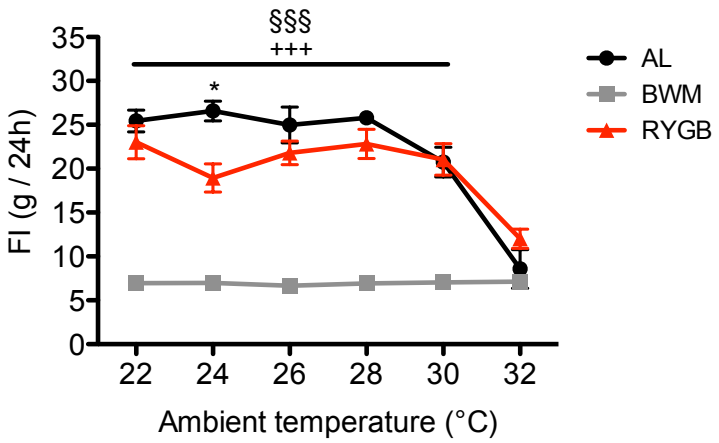

Figure 16 24-hour food intake (FI) of sham operated ad libitum fed (AL, n = 6), sham operated body weight-matched (BWM, n = 5) and gastric bypass operated (RYGB, n = 5) rats at different ambient temperatures. *p < .05 AL vs RYGB; +++p < .001 AL vs BWM; §§§p < .001 BWM vs RYGB. Data represent mean ± SEM.

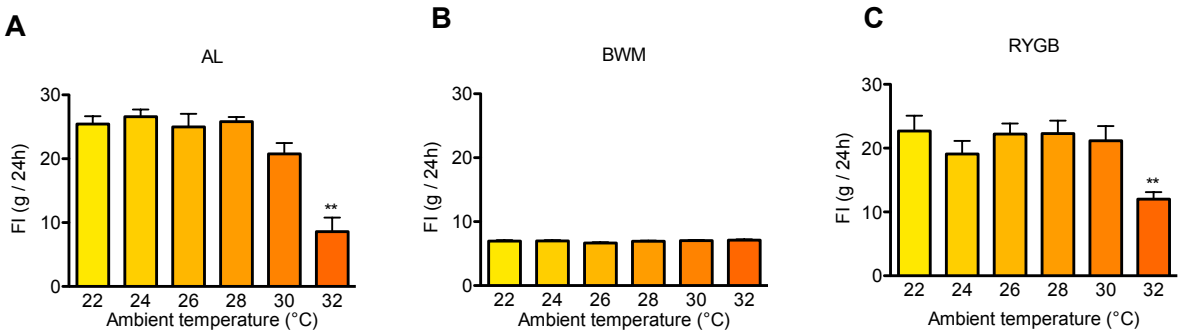

Figure 17 24-hour food intake (FI) of AL (A), BWM (B) and RYGB (C) rats at different ambient temperatures. **p < .01 vs all other ambient temperatures. Data represent mean ± SEM.

Table 5 Mean values ± SEM for food intake of AL, BWM and RYGB rats at different ambient temperatures. See **Figure 16** and **Figure 17** for significances.

	22°C	24°C	26°C	28°C	30°C	32°C
AL	25.44 ± 1.23	26.58 ± 1.13	24.98 ± 2.05	25.81 ± 0.72	20.75 ± 1.70	8.58 ± 2.21
BWM	6.96 ± 0.15	6.98 ± 0.13	6.67 ± 0.11	6.93 ± 0.10	7.04 ± 0.09	7.12 ± 0.11
RYGB	22.70 ± 2.39	19.08 ± 2.07	22.21 ± 1.65	22.29 ± 2.03	21.15 ±2.30	12.03 ±1.10

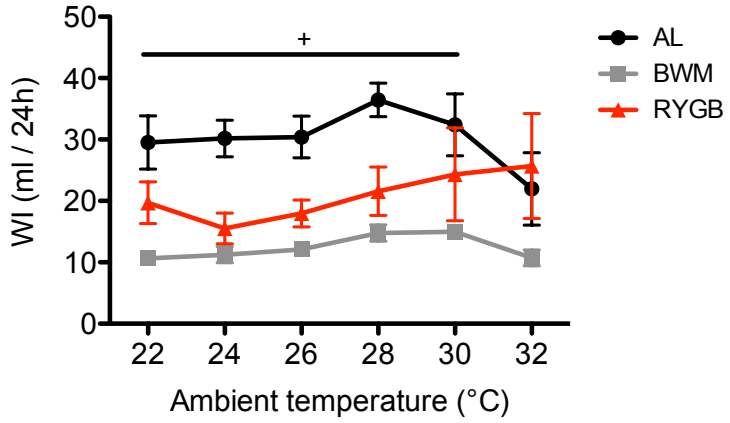

Figure 18 24-hour water intake (WI) of sham operated ad libitum fed (AL, n = 6), sham operated body weight-matched (BWM, n = 5) and gastric bypass operated (RYGB, n = 5) rats at different ambient temperatures. +p < .05 AL vs BWM. Data represent mean ± SEM.

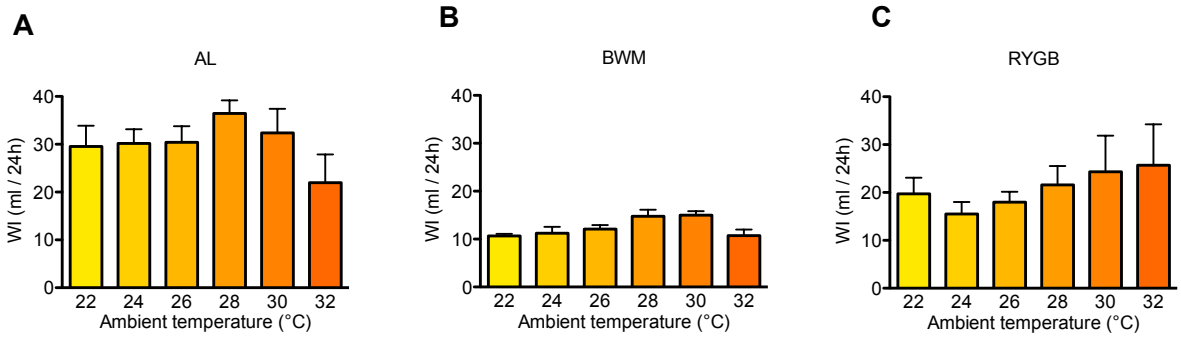

Figure 19 24-hour water intake (WI) of AL (A), BWM (B) and RYGB (C) rats at different ambient temperatures. Data represent mean ± SEM.

Table 6 Mean values ± SEM for water intake of AL, BWM and RYGB rats at different ambient temperatures. See **Figure 18** and **Figure 19** for significances.

	22°C	24°C	26°C	28°C	30°C	32°C
AL	29.53 ± 4.35	30.17 ± 2.98	30.40 ± 3.39	36.45 ± 2.72	32.39 ± 5.04	21.96 ± 5.90
BWM	10.66 ± 0.43	11.24 ± 1.32	12.10 ± 0.84	14.78 ± 1.34	14.99 ± 0.85	10.73 ± 1.28
RYGB	19.71 ± 3.38	15.51 ± 2.51	17.97 ± 2.19	21.57 ± 3.95	24.33 ± 7.56	25.69 ± 8.54

7.2.4. Conclusions

The main goal of this study was to determine and compare the individual TNZ of AL, BWM and RYGB rats. Thermoneutrality is classically defined as the temperature range where the metabolic rate is minimal due the absence of thermoregulatory processes;[43] its identification should therefore in theory be relatively easy. However, our data were not as clear as expected, which complicated the analysis and interpretation. Nevertheless, there are several important and interesting conclusions that can be drawn from the present experiment.

First, the EE data of AL rats were very consistent with the literature on thermoneutrality in freely feeding rats.[75,76] EE was lowest and relatively stable at ambient temperatures between 26 and 30°C, suggesting that this range represents the TNZ of AL rats. EE slowly increased when ambient temperature was lowered to 24°C and was further elevated at 22°C, pointing to a progressive activation of adaptive thermogenesis. In line with this, T_C, food and water intake and physical activity were stable within the 22-30°C range, suggesting that a small increase in adaptive thermogenesis was sufficient to maintain normal metabolic functions at temperature slightly below the TNZ. At an ambient temperature of 32°C, however, EE and T_C increased markedly despite a reduction in food intake. This strongly suggests that a temperature of 32°C was above the UCT; the increase in physical activity, although not significant, further supports the initiation of thermoregulatory behavior.[43] Water intake could be expected to increase when the UCT is reached due to increased evaporative water loss. We do not know why this was not the case in our AL rats, but it has to be mentioned that we did not control for humidity in the current study. It is therefore possible that the increased evaporative water loss led to a higher humidity in the cages, which could have caused the reduction in food and water intake, as well as increased physical activity due to escape behavior. However, although this may be a limitation of this study that needs to be considered for future experiments, it does not contradict the main conclusion that an ambient temperature of 32°C lies above the TNZ of AL rats.

Second, BWM rats seem to have a higher TNZ than AL rats, which is also consistent with the literature showing a fasting-induced shift of about 1°C in the TNZ of rats.[253] This conclusion can be made based on two observations. (1) BWM rats did not show any increase in EE at 32°C compared to lower temperatures and (2) their T_C was almost identical at 30 and 32°C, while there was a progressive increase at lower temperatures up to 30°C. Since the amount of food that the BWM rats received daily was kept constant at all temperatures, their only option to maintain a stable energy balance at lower temperatures was to reduce the T_C. When analyzing T_C at ambient temperatures between 22 and 30°C, there was indeed a very strong correlation between ambient temperature and T_C (R^2 = 0.96, p<0.001), which was not the case in AL rats (R^2 = 0.68, p=0.09). This suggests that only at 30°C, BWM reached an ambient temperature at which there was no necessity for a decreased T_C to compensate for the higher energetic requirement of adaptive thermogenesis.

Third, the RYGB rats are the most difficult to interpret. Even though they showed a similar increase in T_C and decrease in FI as AL rats at 32°C, there was no considerable change in EE at this temperature that would point to an exceeding of the UCT. Based on this it could be speculated that RYGB rats have a very slight shift in their TNZ compared to AL rats, although they also had lowest EE values around 28-30°C. Surprisingly, they did not show an increase in EE at lower temperatures as did the AL rats. One potential interpretation is that the metabolic requirement at temperatures below the LCT, i.e. the requirement of adaptive thermogenesis, is reduced in RYGB

rats. The metabolic requirement at lower temperatures strongly depends on thermal conductance, since higher thermal conductance leads to higher heat loss when ambient temperature decreases. Two major determinants of thermal conductance are body weight and skin blood flow.[43] In rats and mice, the tail is a crucial thermoregulatory organ; the blood flow to the tail is therefore a major determinant of thermal conductance. Thermal conductance has a positive correlation with skin or tail blood flow, but is generally negatively associated with body weight. However, there is also a strong influence of body composition, i.e. the proportion of fat mass. In particular, the thickness of the subcutaneous fat layer has an important influence on body insulation and thereby thermal conductance. The strong impact of body weight on thermal conductance can partly explain why BWM rats may have a higher TNZ than AL rats. However, it does not explain why the metabolic requirement at temperatures below LCT should be smaller in RYGB than in AL rats; on the contrary, the opposite would be expected based on the large differences in body weight. What remains as a potential explanation is therefore that RYGB may have reduced skin blood flow, which would decrease their thermal conductance and thereby their requirement of adaptive thermogenesis. It has indeed been shown in humans that RYGB surgery significantly reduces skin blood flow.[254] Furthermore, measurement of UCP-1 content in BAT of rats kept at room temperature (22°C) revealed lower UCP-1 levels in RYGB than in AL rats (Figure 19, unpublished data), which is also consistent with a lower requirement of adaptive thermogenesis.

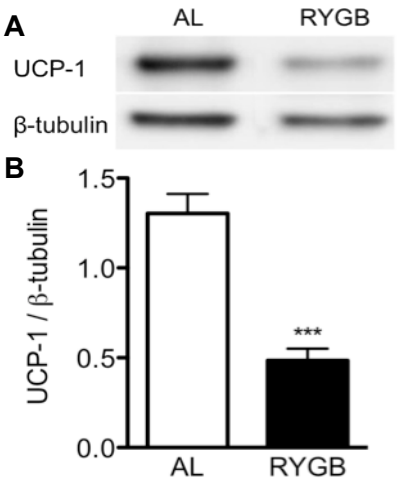

Figure 20 Representative images (A) and quantification (B) of uncoupling protein-1 (UCP-1) content relative to β-tubulin in brown adipose tissue of sham operated ad libitum fed (AL, n=9) and gastric bypass operated (RYGB, n=7) rats fasted for 8 hours at 22°C. ***p < 0.001 vs AL. Data represent mean ± SEM

Finally, with regard to the main question if differences in TNZ led to a false reporting of RYGB-induced alterations in EE, we could clearly show that the differences in EE between BWM and RYGB rats did not disappear at thermoneutrality (Figure 10). On the contrary, in the current study, the difference between RYGB and AL rats that was reported in an earlier study[249] was only statistically significant at temperatures outside the TNZ. This finding should be further investigated by recording EE in RYGB and sham rats at thermoneutrality, ideally directly after surgery before significant weight loss occurs. In addition, it would be of high interest to measure skin blood flow in RYGB and sham rats at different ambient temperatures, or, alternatively, use a heat camera to detect differences in thermal conductance.

8. Further Publications

8.1. Original research article: “Bone-mineral density and expression of vitamin-D receptor dependent calcium uptake mechanisms in the proximal small intestine after Roux-en-Y gastric bypass surgery.”

This section contains an original research article that was submitted for publication to the British Journal of Surgery in October 2013.

My contribution to this manuscript includes data analysis, data interpretation and revising the manuscript.

Bone-mineral density and expression of vitamin-D receptor dependent calcium uptake mechanisms in the proximal small intestine after Roux-en-Y Gastric Bypass Surgery.

Elias, E[1]., Casselbrandt, A[1]., Werling, M[1]., Abegg, K[2]., Laurenius, A[1]., Vincent, R.P[3]., Alaghband-Zadeh, J[3]., Olbers, T[1]., le Roux, C. W[1,3,4]., Fändriks, L[1]., Wallenius, V[1]*.

[1]Dept of Gastrosurgical Research and Education, Sahlgrenska Academy, University of Gothenburg, Sweden. [2]Institute of Veterinary Physiology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland. [3]Department of Clinical Biochemistry, King's College Hospital NHS Foundation Trust, London, UK. [4]Diabetes Research Centre, UCD Conway Institute, School of Medicine and Medical Science, University College Dublin, Ireland.

Short title: Decreased BMD after Roux-en-Y Gastric Bypass.

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List of abbreviations

RYGBP = Roux-en-Y Gastric Bypass

VBG = Vertical Banded Gastroplasty

BMI = Body mass index (kg/length2)

BMD = Bone mineral density (gram/cm2)

VDR = vitamin-D receptor

ABSTRACT

Context: Bypass of the proximal small intestine after Roux-en-Y Gastric Bypass (RYGBP) surgery may lead to decreased calcium uptake and possibly osteopenia.

Objectives: To examine the operation-specific effects of RYGBP on bone mineral density (BMD) and to explore potential mechanisms for decreased calcium uptake after RYGBP.

Methods: Patients randomized to either RYGBP or vertical banded gastroplasty (VBG) were compared. Dual-energy X-ray absorptiometry (DXA) data were analyzed for operation-specific changes in total BMD and non-weight bearing (skull) BMD up to six years after surgery. These patients were not routinely supplemented with calcium or vitamin-D. Bone resorption markers in serum and calcium uptake mechanisms in the jejunal mucosa after RYGBP surgery were compared at baseline and post-operatively.

Results: Postoperative weight loss was similar in RYGBP and VBG patients during the first year. Skull BMD was decreased in the RYGBP patients ($P<0.001$), but not after VBG. During the weight stable phase, from one to six years postoperatively, the RYGBP patients, but not VBG patients, continued to decrease in skull BMD as well as total BMD. In line with the BMD decrease, serum C-terminal telopeptide (CTX) levels increased at 18 months postoperatively in RYGBP patients. Proteomic analysis of the jejunal Roux-limb mucosa in RYGBP patients revealed a decrease in the levels of Heat shock protein (Hsp) 90β, a co-activator of the vitamin-D receptor (VDR). Despite increased VDR levels, the levels of the VDR-regulated calcium transporter protein TRPV6 were also decreased.

Conclusion: RYGBP is associated with a decrease in BMD at one to six years after surgery, in contrast to VBG. This could be attributed to impaired active calcium absorption caused by a combination of the duodenal bypass and decreased activation of components of the vitamin-D dependent calcium absorption mechanisms including Hsp90β and TRPV6.

INTRODUCTION

Bariatric surgery is currently the most effective treatment of obesity. Roux-en-Y Gastric Bypass (RYGBP) has been shown to reduce mortality and obesity-related metabolic complications compared to non-surgical obesity treatment (1).

Although calorie malabsorption is not believed to be the primary mechanism explaining the weight loss after RYGBP, there is some concern regarding the risk of a decreased uptake of specific vitamins and minerals due to the rerouting of the proximal gastrointestinal tract. RYGBP results in exclusion of the major part of the stomach and the entire duodenum as well as diversion of bile and pancreatic juices from the alimentary-limb, i.e. the proximal jejunum. Uptake of certain nutrients that are primarily absorbed in the proximal small intestine could therefore be affected.

Intestinal calcium uptake is dependent on the amount of calcium ingested and the efficiency of intestinal calcium uptake (3). Intestinal calcium uptake is partly an active transcellular process, which is saturable, and partly a passive paracellular process that is non-saturable (3). The active calcium absorption is an energy dependant process that is localised to the proximal small intestine, i.e. duodenum and proximal jejunum (3). The efficiency of active calcium transportation is regulated by activated vitamin-D via the vitamin-D receptor (VDR) (3). The exact mechanism by which VDR regulates calcium uptake is unclear but several studies have shown that VDR activated by vitamin-D, transcriptionally regulates several proteins believed to participate in active calcium absorption, such as the calcium transporter protein TRPV6 (4, 5).

We hypothesized that the exclusion of the duodenum and the diversion of bile and pancreatic juices from the proximal jejunum could affect intestinal calcium absorption and possibly result in a decreased bone mineral density (BMD). As RYGBP induces weight loss that by itself may affect whole body BMD we also studied non weight-bearing skull BMD. Further, we measured pre- and 18 month postoperative levels of bone resorption marker C-terminal telopeptide (CTX), parathyroid hormone (PTH), 25 (OH) vitamin D, 1,25 (OH)2 vitamin D and vitamin D binding protein in RYGBP patients.

We performed a proteomics analysis of the alimentary-limb jejunal mucosa before and after RYGBP to identify changed protein expression levels that could potentially affect VDR activity. We

also examined expression levels of proteins regulated by VDR to find and evaluate possible changes in VDR activity after RYGBP.

MATERIALS AND METHODS

Study outline

As described previously (6-8), 82 patients were randomized in a clinical trial comparing gastric bypass (n=37) and VBG (n=45). Both procedures were performed laparoscopically according to previously described surgical techniques. Blood samples for bone resorption markers from RYGBP operated patients were retrieved at baseline and 18 months after the operation in another study setting where 63 patients (43 females and 20 men) were recruited for standard meal tests before and 18 months after surgery. Only preoperative and 18 month fasting baseline samples were used for the analysis of bone resorption markers in the present study. The local ethics committee of the University of Gothenburg approved the study protocols (reference numbers 380-06 and 583-07) and the studies were conducted according to the principles of the Helsinki declaration. All patients gave written informed consent. All randomized patients were invited to a clinical follow up at 12, 18 months and six years after surgery, which included measurement of body weight, body composition, blood sampling and eating habits. In a small number of the patients gastroscopy was performed at 6-8 months postoperatively for sampling of jejunal mucosa for proteomic analysis.

Weight, height, BMI and body composition

Weight and height were measured with the patient in light underwear after an overnight fast. Height was measured to the closest 0.5 cm using a wall mounted standard stadiometer with the patient in a standing position. Weight was measured to the nearest 0.1 kilogram on an electronic scale, which was calibrated at regular intervals. Body mass index (BMI kg/m2) was calculated as kilogram body weight divided by height square meters. Weight outcome at two years postoperatively has been previously reported from this study (6).

Body composition was assessed in patients with primary gastric bypass (n=28, 0-1 year postoperatively and n=17, 1-6 years postoperatively) and VBG (n=35, 0-1 year postoperatively and n=14, 1-6 years postoperatively) using a LUNAR DPX-IQ DXA scanner (Lunar Co., Madison WI). Software version 4.7, 4.7c or 4.7e was used at baseline and version 4.7e at the 6 year follow up. An extended analysis program for total body analysis (LUNAR Radiation, Madison, WI) was used. Body fat, lean tissue mass, bone mineral content, bone mineral density and body weight were assessed as previously described (9)

Dietary intake

All patients were prescribed a multi-vitamin and mineral supplementation after primary surgery and patients in the gastric bypass group were additionally prescribed a supplementation of vitamin B12. When the study commenced calcium and vitamin-D supplementation was not prescribed routinely. Therefore, patients in the randomized controlled clinical trial used for analysis of BMD did not systematically receive calcium and vitamin-D supplementation. However, the patients in the second study recruited for standard meal tests were prescribed calcium/vitamin-D supplementation (500 mg/400 IE, twice daily).

Patients were asked to fill in a recall questionnaire in which they were instructed to describe their food intake over the previous three months. The questionnaire has been validated for assessment in obese and lean subjects. Total energy intake, intake of macronutrients (fat, carbohydrates and protein) and different food groups (e.g. fruit and vegetable, desserts, candy or prepared food) were analyzed from the questionnaire (10) .

Measurement of bone resorption markers in serum

25 (OH) vitamin-D was measured by Liaison® (DiaSorin.Inc, USA). The reference range for 25 (OH) vitamin-D was 25-100 nmol/L. 1,25-dihydroxy vitamin-D (1,25(OH)2D) was measured using a commercially available radioimmunoassay kit (AA-54F, Immunodiagnostic Systems Ltd., Baldon, UK). The intra- and inter-assay CVs were 8.7% (n=20) and 12.6% (n=20), respectively. The 95% CI for normal adults for the 1,25 assay was 43-168 pmol/L. Intact PTH (iPTH) was measured by

ADVIA Centaur® (SIEMENS Healthcare Diagnostic Ltd, UK). Interassay CV for PTH was 3.4-5.2% and intraassay CV was 4.3-9.1%. The reference range for PTH was 10-70 ng/ml. Serum CrossLaps® ELISA kit (IDS Ltd, UK) was used for the measurement of CTX, with an interassay CV of <6% and an intraassay CV of <10 %. The reference range for CTX was: pre-menopausal women = 0.122-0.738 ng/ml (95% CI; mean value 0.287); post-menopausal women = 0.142-1.351 ng/ml (mean 0.439). Quantakine® ELISA kit (R&D Systems, USA) was used to measure vitamin-D binding protein.

Protein expression in the alimentary-limb mucosa after RYGBP

For the proteomics analysis, tissue samples were collected as previously described (11). In short, samples were collected from eight patients that went through a first time laparoscopic RYGBP or a conversion from VBG to RYGBP. The operative technique included an antecolic-antegastric Roux-en-Y construction with a 10 to 20 ml gastric pouch. The gastro-entero- anastomosis was constructed with a straight 45 mm stapler and complementary hand-suturing. A tissue sample was removed from the jejunum between the gastro-entero and the entero- entero anastomosis as the 75 cm loop was divided to create the Roux-en-Y construction. After excision the mucosa/submucosa was separated from the musculature by means of sharp dissection. Between six to eight months after surgery the patients underwent an endoscopic examination of the alimentary-limb. Mucosal biopsies were collected approximately 8 cm distal to the stoma. Mucosal tissue specimens were snap-frozen in liquid nitrogen and kept frozen (-70°C) for later analysis of protein expression. Global protein expression analysis of proximal jejunal mucosal epithelium was performed by 2-D gel electrophoresis and mass spectrometry by nanoflow LC-MS/MS as described elsewhere (manuscript in preparation). Only proteins that were regulated in the same direction in all patients (N=8), and at least 50% from baseline were selected for further analysis.

Western blot

Total protein samples were diluted in SDS buffer and heated at 70˚C for 10 min. before they were loaded on a NuPage 10 % Bis-Tris gel, and electrophoresis run using a MOPS buffer (Invitrogen AB, Lidingo, Sweden). One lane of each gel was loaded with a prestained molecular weight standard (SeeBlue, Invitrogen AB, Lidingo, Sweden). A positive control was loaded when available. After the electrophoresis the proteins were transferred to a polyvinyldifluoride transfer membrane, Hybond, 0.45µm, RPN303F, (Amersham, Buckinghamshire, UK) using an iBlot (Invitrogen AB, Lidingö, Sweden). Membranes were then incubated with polyclonal specific primary antibodies against Hsp90β, VDR or TRPV6. A secondary alkaline phosphatase conjugated goat anti-rabbit IgG antibody was used with CDP-Star (Tropix, Bedford, MA, USA) as a substrate, to identify immunoreactive proteins by chemiluminescense. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, IMG-5143A, Imgenex, BioSite, San Diego, CA, USA) was used as control for equal loading and for each tested sample the optical density of primary antibody/GAPDH represents the result. The membrane was stripped for reprobing with other primary antibodies using a stripping buffer (Re-Blot Plus Mild Solution (10X), Millipore, Temecula, CA, USA). Images were captured by a Chemidox XRS cooled CCD camera, and semi-quantification was performed using Quantity One software (Bio-Rad Laboratories, Hercules, USA).

Statistical analyses

Statistical analyses of DXA data and Western blot data were performed using SPSS v.16 and non-parametric tests, i e Wilcoxon sign rank test and Mann–Whitney U. A multiple linear regression model was used to evaluate impact of operation type and weight loss with regards to changes in skull BMD from baseline to year one. Paired-samples t-test was used for analysis of intra-individual changes in hormone levels of CTX, 25 (OH) vitamin D, 1,25 (OH)2 vitamin D, PTH and vitamin D-binding protein, as well as for the analysis of changes of the protein levels in the proteomics analysis of the spot intensities on the 2D-gel electrophoreses.

RESULTS

Baseline data

Baseline statistics have previously been presented in earlier publications (6, 7). In short, there were no statistical differences between preoperative BMI, age, total BMD, sex ratio and smoking.

Weight loss phase, zero to one year postoperatively

Weight baseline to one year postoperatively

At one year postoperatively, body weight was significantly lower in the RYGBP vs. the VBG operated patients (Table 1). The weight loss (preop weight-postop weight) was however not significantly different between the groups but both groups decreased significantly in weight (Table 1).

Total BMD baseline to one year postoperatively

There were no significant differences in total BMD prior to surgery (Table 1). At one year postoperatively, there was no significant difference between the groups in total BMD or in the change of total BMD (Table 1). VBG operated patients had a small but significant increase in total BMD (Table 1).

Skull BMD baseline to one year postoperatively

There was no significant difference in skull BMD at baseline or at one year postoperatively between the RYGBP and the VBG patients (Table 1). In the RYGB operated patients, but not in the VBG patients, skull BMD was however significantly reduced at one year compared to baseline (Table 1). Delta skull BMD (skull BMD pre-op – skull BMD post-op) in the RYGBP operated patients after one year was also significantly different compared to the VBG patients (Table 1).

Multiple linear regression analysis

In a multiple linear regression model analyzing factors predicting changes in skull BMD during the first year postoperatively, operation type (VBG or RYGBP) had significant influence on the changes in skull BMD (P=0,002), whereas the weight loss (delta weight 0 to1 years postoperatively) did not have a significant influence (P= 0,21).

Weight stable phase, one to six years postoperatively

Weight one to six years postoperatively

RYGBP patients did not change significantly in weight during the period from one to six years (Table 2). The VBG patients had significantly higher body weight at six years compared to the RYGBP patients (Table 2). Although VBG operated patients had a greater weight at six years postoperatively, there was neither a statistically significant increase in weight within the VBG group nor between the groups (Table 2).

BMD one to six years postoperatively

Postoperatively, in the weight-stable phase from years one to six, total body BMD decreased significantly in the RYGBP group (Table 2). Patients operated with VBG also decreased slightly in total BMD from year one to six but still had significantly higher BMD compared to the RYGBP patients at six years postoperatively (Table 2). The RYGBP operated patients had a larger decrease of BMD years one to six compared to the VBG operated patients (Table 2).

Skull BMD one to six years postoperatively

During years one to six, the RYGBP operated patients continued to decrease in skull BMD (Table 2). Skull BMD was significantly lower at six years compared to one year postoperatively, and delta skull BMD was significantly decreased in the RYGBP patients (Table 2). There was no statistically significant change in skull BMD in the VBG operated patients from year one to six (Table 2).

Calcium intake at baseline, one and six years after surgery

Dietary questionnaires were sent out to all patients that were randomized to VBG or RYGBP and deemed eligible for inclusion, (n= 50, 50) preoperatively and one year post-operatively. After six years dietary questionnaires were sent out to patients that were included in the study and remained in their original group.

Pre-operatively, patients in the RYGBP group reported a mean daily calcium intake of 1,5 ± 0,8 g (mean ± SD; n=43), patients in the VBG group reported a mean daily calcium intake of 1,9 ± 0,9 g (n=48) One year postoperatively patients in the RYGBP group reported a mean daily calcium intake of 1,0 ± 0,5 g (n=46), patients in the VBG group reported a mean daily calcium intake of 1,3 ± 0,7 g (n=44).

After six years patients in the RYGBP group reported a mean daily calcium intake of 1,1 ± 0,4 g (n=9), patients in the VBG group reported a mean daily calcium intake of 2,0 ± 0,8 g (n=7).

Bone resorption markers at baseline and 18 months after RYGBP surgery

CTX levels were significantly increased from baseline to 18 months post-operatively (table 3). Both 25 (OH) vitamin-D and 1,25 (OH)2 vitamin-D also increased from baseline to post-operatively (table 3). The ratio between 25 (OH) vitamin-D and 1,25 (OH)2 vitamin-D was however, not changed (table 3).

There was also a small but significant decrease in PTH from baseline to post-operatively (table 3). Vitamin-D binding protein was not changed (table 3).

Changed protein expression in the Roux-limb

Proteomic analysis.

In order to explore the mechanisms for the potentially decreased active calcium uptake in the proximal jejunum, global protein expression analysis of jejunal biopsies preoperatively and six to eight months postoperatively after RYGBP surgery was performed. This showed, among several other protein regulations, a significant decrease in signal intensity in a spot that mass spectroscopy identified as containing Hsp90β (average ratio -1,64, $P<0,01$, N=8). After searching relevant

literature, this was the only protein change identified that was found to be related to active intestinal calcium absorption.

VDR and VDR-associated proteins assessed by Western blots.

Hsp90β

Western blot analysis of paired intra-individual jejunal mucosal samples before and after RYGBP from 10 additional patients analyzed in a similar setting confirmed a significant decrease in Hsp 90β levels after RYGBP (Figure 1)

TRPV6.

Protein expression levels of the calcium transporter protein TRPV6 in the jejunum decreased after RYGBP (Figure 2). Changes were analyzed intra-individually within the same patient before and after surgery. The change in TRPV6 levels was significantly correlated with the change in Hsp 90β levels ($R=0.78$, $P<0.01$, $N=10$; Figure 3).

VDR

Jejunal VDR protein levels increased significantly after RYGBP (Figure 4).

DISCUSSION

Several previous studies have indicated reduced intestinal calcium absorption after RYGBP surgery (12, 13). Insufficient calcium uptake could eventually lead to demineralization of the skeleton, subsequent osteoporosis and increased fracture risk (14, 15). In the present article, we aimed to characterize changes in BMD after two mechanistically different bariatric procedures, RYGBP and VBG. After RYGBP, the foregut is excluded from passage of ingested food, and bile is diverted from the majority of the jejunum. We therefore hypothesized that calcium uptake through

the vitamin-D dependent active transport mechanisms in the proximal small intestine might be affected by RYGBP, but not by VBG.

We describe the effects of RYGBP and VBG on BMD in both the weight-loss phase (0 to one years post-operatively) and the long-term weight-stable phase (one to six years postoperatively). As weight loss induced by RYGBP may influence BMD we compared patients that were randomized to RYGBP or to VBG operations. We also analyzed skull BMD as a non weight-bearing bone that should not be affected by weight loss per se.

We show that RYGBP specifically induces a reduction in non-weight-bearing skull BMD during the first year after surgery and that RYGBP seems to have a long-term impact also on total body BMD at six years after the operation. This was not seen in patients that underwent VBG surgery, but had a similar weight change during the first six years postoperatively. The bone resorption marker CTX was prominently increased after RYGBP surgery. Further, we describe a changed expression pattern of key proteins involved in the active calcium uptake in the proximal small intestine after RYGBP surgery.

The strengths of this article are that the patient data on RYGBP vs. VBG originates from a randomized controlled clinical trial and that the patients did not receive oral calcium supplementation, which was not routine at our center in 1999 when the study was initiated. Calcium and vitamin-D is now routinely prescribed postoperatively to all bariatric patients. Weaknesses include the lack of blood samples from the randomized controlled clinical trial and relatively short follow up time of only six years post-operatively. A large number of patients originally assigned to VBG had been converted to RYGBP at the six-year follow-up. Only patients that remained in their original group per protocol were analyzed at six years, hence the lesser number of subjects at this time point. Therefore, intention to treat group analysis of osteoporotic fracture risk was not meaningful. Further, a weakness is that we were not able to measure intestinal calcium uptake per se, and we are not aware of a method for this that is useful in the clinical setting of this study.

Within one year postoperatively, weight loss induced by the two procedures did not significantly differ, yet BMD data showed two distinct patterns. The group that underwent VBG did not decrease their skull BMD and somewhat surprisingly even increased slightly in total BMD, possibly reflecting increased physical activity after the rapid weight loss during the first post-operative year. On the other hand, the RYGBP operated patients did not show an increase in total BMD, but showed a significant decrease in skull BMD, as early as one year post-operatively (Table 1).

As previously mentioned, we analyzed skull BMD as a non-weight bearing bone that would not be affected by weight loss, and this was supported using a multiple linear regression model that indicated that the type of operation rather than weight loss predicted the changes in skull BMD.

During the weight-stable period one to six years post-operatively, RYGBP operated patients where weight stable, however they continued to decrease their skull BMD and also decreased their total BMD (Table 2).

Data from dietary questioners were used to calculate calcium intake. These data indicate that calcium intake at all times was well above the recommended daily intake of calcium for both groups (Livsmedelsverket, Uppsala, Sweden).

Since we did not have blood samples left from the first series of RYGBP and VBG patients, we analyzed bone resorption markers in blood samples from another series of RYGBP patients operated at our center. The difference between these studies was that in this latter series all patients were prescribed calcium and vitamin-D supplements (500 mg/400 IE, twice daily).

We found that most profoundly CTX levels in serum doubled at 18 months postoperatively. This should reflect increased bone turnover and may well be in line with increased bone resorption and the decreased BMD levels in the first series of patients. Alternatively, it could merely be a response to the decreased bodyweight leading to increased bone turnover, although the lack of BMD decrease in the VBG patients, with similar weight loss during the first year postoperatively is not consistent with increased bone turnover as a response primarily to the weight loss per se. Skull BMD as a non-weight bearing part of the skeleton should also not decrease due to decreased body weight. Further, in the first series of RYGBP, but not VBG patients we saw a continued decrease of

BMD six years post-operatively. At that time, body weight had stabilized and was not further decreased compared to one year post-operatively.

25 (OH) and 1,25 (OH)2 vitamin-D levels increased postoperatively compared to baseline, and were high compared to the reference range, probably reflecting the vitamin-D substitution therapy given to these patients. The ratio between 25 (OH) and 1,25 (OH)2 vitamin-D levels were however not changed, suggesting an unchanged 1α-hydroxylase activity.

We also found a small but significant decrease of PTH at 18 months postoperatively. The decreased PTH levels could be a response to the increased vitamin-D levels. This may reflect a non-functional regulation since vitamin-D signaling in the proximal small intestine seemed to be decreased based on the down-regulations of Hsp90β and TRPV6, despite increased VDR levels. Other explanations are also possible, as it has been reported that obese patients in general tend to have low vitamin-D levels, and these seem to be normalized postoperatively (16, 17). The decreased PTH levels could be a secondary response to that.

Our data suggests that calcium absorption could be affected by RYGBP and based on previous research hypothesized that it is a consequence of impaired active intestinal calcium absorption in the proximal small intestine.

As previously mentioned, active intestinal calcium absorption is located in the proximal intestine (3) and the exclusion of the duodenum will negate calcium uptake there. There is also a possibility that the diversion of bile from the proximal jejunum i.e. the alimentary-limb might inhibit normal calcium absorption (18).

Therefore, we also performed a proteomic analysis of jejunal mucosa before and after RYGBP to determine changes in protein expression levels that might influence active intestinal calcium absorption. Among the regulated proteins was Hsp90β that has been reported to influence VDR activity (19). Western blot analysis confirmed decreased levels of Hsp90β in alimentary-limb mucosa after RYGBP (Figure 1). We then chose to measure protein levels of TRPV6 as a marker for VDR activity. TRPV6 has previously been shown to be transcriptionally regulated by VDR in the human proximal intestine (4). As TRPV6 was significantly decreased (Figure 2) we suggest that this reflects a decrease in VDR activity after RYGBP caused by the reduced Hsp90β levels. There

was also a significant correlation between the decrease in Hsp90β and TRPV6 (Figure 3). The increase in VDR expression levels may reflect a compensatory response that could result from insufficient calcium absorption (Figure 4). A hypothetical figure for the changes of active vitamin-D receptor mediated calcium uptake mechanisms after RYGBP is provided in Figure 5.

A large proportion of patients undergoing RYGB receive oral calcium supplementation as well as vitamin-D substitution. However, if intestinal vitamin-D induced uptake of calcium is impaired, oral supplementation could be insufficient. There are recent data indicating that calcium uptake may be decreased after RYGBP, even with calcium and vitamin-D supplementation, and lead to increased PTH activity (12). A recent publication showed that RYGBP patients are at increased risk for recalcitrant symptomatic hypocalcemia after thyroidectomy (20). This is well in line with our present findings.

Taken together, we have demonstrated a decrease in BMD (weight bearing and non-weight bearing) over time in non-supplemented RYGBP patients. We suggest a hypothesis of a decrease in proximal small intestinal active calcium uptake after RYGBP. This calls for long-term studies on risk of osteoporosis after RYGBP surgery also exploring whether calcium and vitamin-D supplementation can abolish this decline.

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REFERENCES:

1. Sjostrom L, Narbro K, Sjostrom CD, Karason K, Larsson B, Wedel H, et al. Effects of bariatric surgery on mortality in Swedish obese subjects. N Engl J Med 2007;357(8):741-52.

2. Schauer PR, Kashyap SR, Wolski K, Brethauer SA, Kirwan JP, Pothier CE, et al. Bariatric surgery versus intensive medical therapy in obese patients with diabetes. N Engl J Med;366(17):1567-76.

3. Fleet JC, Schoch RD. Molecular mechanisms for regulation of intestinal calcium absorption by vitamin D and other factors. Crit Rev Clin Lab Sci;47(4):181-95.

4. Balesaria S, Sangha S, Walters JR. Human duodenum responses to vitamin D metabolites of TRPV6 and other genes involved in calcium absorption. Am J Physiol Gastrointest Liver Physiol 2009;297(6):G1193-7.

5. Lieben L, Benn BS, Ajibade D, Stockmans I, Moermans K, Hediger MA, et al. Trpv6 mediates intestinal calcium absorption during calcium restriction and contributes to bone homeostasis. Bone;47(2):301-8.

6. Olbers T, Bjorkman S, Lindroos A, Maleckas A, Lonn L, Sjostrom L, et al. Body composition, dietary intake, and energy expenditure after laparoscopic Roux-en-Y gastric bypass and laparoscopic vertical banded gastroplasty: a randomized clinical trial. Ann Surg 2006;244(5):715-22.

7. Olbers T, Fagevik-Olsen M, Maleckas A, Lonroth H. Randomized clinical trial of laparoscopic Roux-en-Y gastric bypass versus laparoscopic vertical banded gastroplasty for obesity. Br J Surg 2005;92(5):557-62.

8. Werling M, Fandriks L, Bjorklund P, Maleckas A, Brandberg J, Lonroth H, et al. Long-term results of a randomized clinical trial comparing Roux-en-Y gastric bypass with vertical banded gastroplasty. Br J Surg;100(2):222-30.

9. Kullberg J, Brandberg J, Angelhed JE, Frimmel H, Bergelin E, Strid L, et al. Whole-body adipose tissue analysis: comparison of MRI, CT and dual energy X-ray absorptiometry. Br J Radiol 2009;82(974):123-30.

10. Lindroos AK, Lissner L, Sjostrom L. Does degree of obesity influence the validity of reported energy and protein intake? Results from the SOS Dietary Questionnaire. Swedish Obese Subjects. Eur J Clin Nutr 1999;53(5):375-8.

11. Spak E, Bjorklund P, Helander HF, Vieth M, Olbers T, Casselbrant A, et al. Changes in the mucosa of the Roux-limb after gastric bypass surgery. Histopathology;57(5):680-8.

12. Hewitt S, Sovik TT, Aasheim ET, Kristinsson J, Jahnsen J, Birketvedt GS, et al. Secondary Hyperparathyroidism, Vitamin D Sufficiency, and Serum Calcium 5 Years After Gastric Bypass and Duodenal Switch. Obes Surg.

13. Riedt CS, Brolin RE, Sherrell RM, Field MP, Shapses SA. True fractional calcium absorption is decreased after Roux-en-Y gastric bypass surgery. Obesity (Silver Spring) 2006;14(11):1940-8.

14. Marshall D, Johnell O, Wedel H. Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. BMJ 1996;312(7041):1254-9.

15. Nakamura KM, Haglind EG, Clowes JA, Achenbach SJ, Atkinson EJ, Melton LJ, 3rd, et al. Fracture risk following bariatric surgery: a population-based study. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 2013.

16. Beckman LM, Earthman CP, Thomas W, Compher CW, Muniz J, Horst RL, et al. Serum 25(OH) Vitamin D Concentration Changes After Roux-en-Y Gastric Bypass Surgery. Obesity 2013.

17. Coupaye M, Breuil MC, Riviere P, Castel B, Bogard C, Dupre T, et al. Serum vitamin D increases with weight loss in obese subjects 6 months after Roux-en-Y gastric bypass. Obesity surgery 2013;23(4):486-93.

18. Kehayoglou AK, Williams HS, Whimster WF, Holdsworth CD. Calcium absorption in the normal, bile-duct ligated, and cirrhotic rat, with observations on the effect of long- and medium-chain triglycerides. Gut 1968;9(5):597-603.

19. Angelo G, Lamon-Fava S, Sonna LA, Lindauer ML, Wood RJ. Heat shock protein 90beta: a novel mediator of vitamin D action. Biochem Biophys Res Commun 2008;367(3):578-83.

20. McKenzie TJ, Chen Y, Hodin RA, Shikora SA, Hutter MM, Gaz RD, et al. Recalcitrant hypocalcemia after thyroidectomy in patients with previous Roux-en-Y gastric bypass. Surgery 2013.

Figure legends

Figure 1. Western blot of Hsp90β (A) and GAPDH (B) protein expression levels in jejunal mucosa before and after RYGBP. All samples were paired, i e from same individual before and after surgery. The paired samples are divided by the dotted lines. Grey boxes/bars show baseline samples and white boxes/bars show 6-8 month postoperative samples. * $P<0.05$ between preoperative and postoperative samples.

Figure 2. Western blot of calcium channel protein TRPV6 (A) and GAPDH (B) protein expression levels in jejunal mucosa before and after RYGBP. All samples were paired, i e from same individual before and after surgery. The paired samples are divided by the dotted lines. Grey boxes/bars show baseline samples and white boxes/bars show 6-8 month postoperative samples. ** $P<0.01$ between preoperative and postoperative samples.

Figure 3. Correlation analysis between Δ TRPV6 vs. Δ Hsp 90β protein expression levels measured by Western blot.

Figure 4. Western blot of vitamin-D receptor (VDR; A) and GAPDH (B) protein expression levels in jejunal mucosa before and after RYGBP. All samples were paired, i e from same individual before and after surgery. The paired samples are divided by the dotted lines. Grey boxes/bars show baseline samples and white boxes/bars show 6-8 month postoperative samples. ** $P<0.01$ between preoperative and postoperative samples.

Figure 5. Schematic presentation of the hypothetic regulation of the active vitamin D-induced calcium absorption mechanisms in the proximal jejunum postoperatively after Roux-en-Y gastric bypass surgery: Hsp90β that acts as a co-activator of VDR is decreased. VDR transcriptionally regulates several proteins believed to participate in active calcium absorption, such as the calcium

transporter protein TRPV6. Despite increased VDR expression, TRPV6 expression is decreased, and may ultimately lead to decreased calcium uptake and decreased BMD.

Table 1. Body weight and BMD during weight-loss phase baseline to one year postoperatively.

		Baseline	Postop 1 yr	Δ Postop 1 yr (vs baseline)
Body weight (kg)	RYGBP, n=28	114.9 ± 10.40	80.9 ± 10.2	-33.9 ± 7.6 ###
	VBG, n=35	118.3 ± 9,93	89.7 ± 13.2 **	-28.6 ± 11.0 ###
BMD (g/cm^2)	RYGBP, n=28	1.23 ± 0.06	1.24 ± 0.08	0.0053 ± 0.053
	VBG, n=35	1.23 ± 0.08	1.26 ± 0.07	0.0215 ± 0.036 ##
Skull BMD (g/cm^2)	RYGBP, n=28	2.40 ± 0.22	2.32 ± 0.25	-0.083 ± 0.096 ### **
	VBG, n=35	2.41 ± 0.20	2.41 ±0.20	-0.00046 ± 0.080

P<0.01 and ### P<0.001 within group, baseline vs. postop year one or Δ baseline-postop year one.
** P<0.01 between groups, RYGBP vs. VBG.

Table 2. Body weight and BMD during weight-stable phase one to six years postoperatively

		Postop 1 year	Postop 6 year	Δ Postop 6 yrs (vs postop 1 yr)
Body weight (kg)	RYGBP, n=17	81.8 ± 7.9	84.6 ± 12.1	2.28 ± 10.6
	VBG, n=14	87.4 ± 13.0	97.3 ± 14.5 **	9.58 ± 15.4
BMD (g/cm^2)	RYGBP, n=17	1.24 ± 0.08	1.15 ± 0.07 ##	-0.08 ± 0.06 ## *
	VBG, n=14	1.28 ± 0.07	1.27 ± 0.08 # **	-0.029 ± 0.047 #
Skull BMD (g/cm^2)	RYGBP, n=17	2.37 ± 0.23	2.26 ± 0.20 ##	-0.12 ± 0.133 ##
	VBG, n=14	2.39 ± 0.22	2.38 ± 0.23	-0.051 ± 0.139

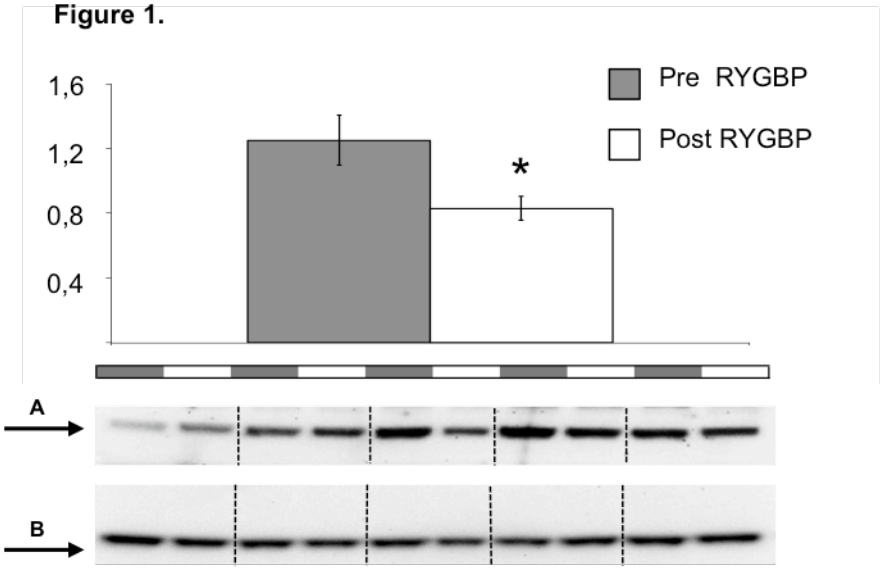
# P<0.05 and ## P<0.01 within group, postop one vs. six years, or Δ one-six years postoperatively.
* P<0.05 and ** P<0.01 between groups, RYGBP vs. VBG.

Table 3. Bone resorption markers before and 18 months after RYGBP surgery

		Baseline (mean ± SD)	Postop 18 months (mean ± SD)	Reference range
BMI (kg/m2)	n=46	44.0 ± 5.8	30.3 ± 6.1 ###	18-25
CTX (pg/ml)	n=39	363 ± 165	811 ± 331 ###	see M&M
PTH (pg/ml)	n=37	63.4 ± 36	52.1 ± 29 #	10-70
25 (OH) Vitamin D (nmol/L)	n=38	38.9 ± 19	58.6 ± 18 ###	25-100
$1,25\ (OH)_2$ Vitamin D (pmol/L)	n=37	135 ± 49	231 ± 98 ###	43-168
Ratio 25 D/1,25 D (pmol/pmol)	n=37	281 ± 194	258 ± 135	-
Vitamin D binding protein (µg/ml)	n=38	290 ± 104	334 ± 122	-

P < 0.05, # # # P < 0.001 baseline vs. 18 mon postoperatively.
No significant differences were found between the genders, except for 25 (OH) vitamin D

Figure 1.



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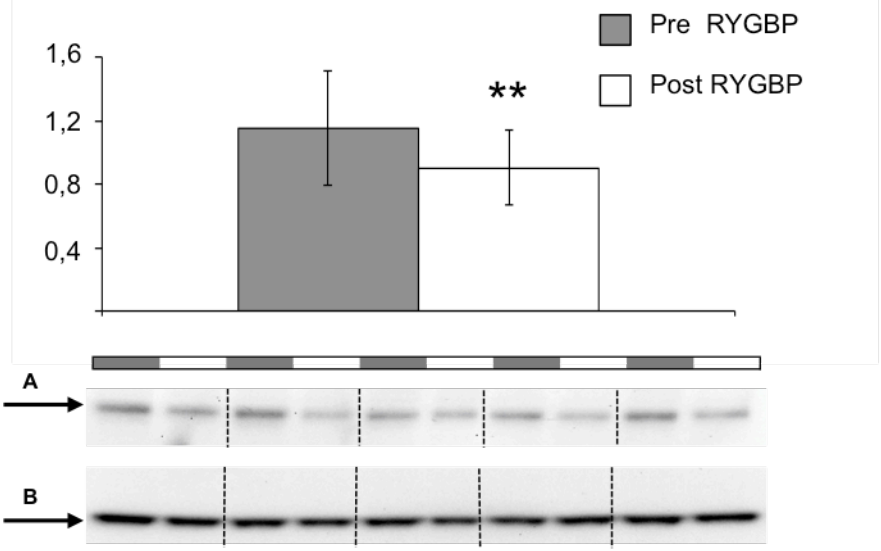
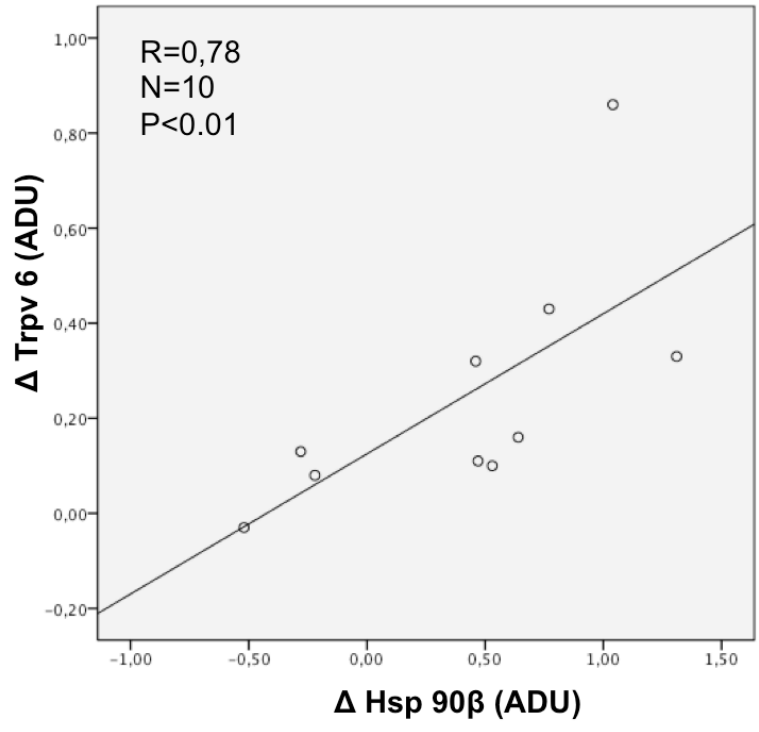


Figure 3.



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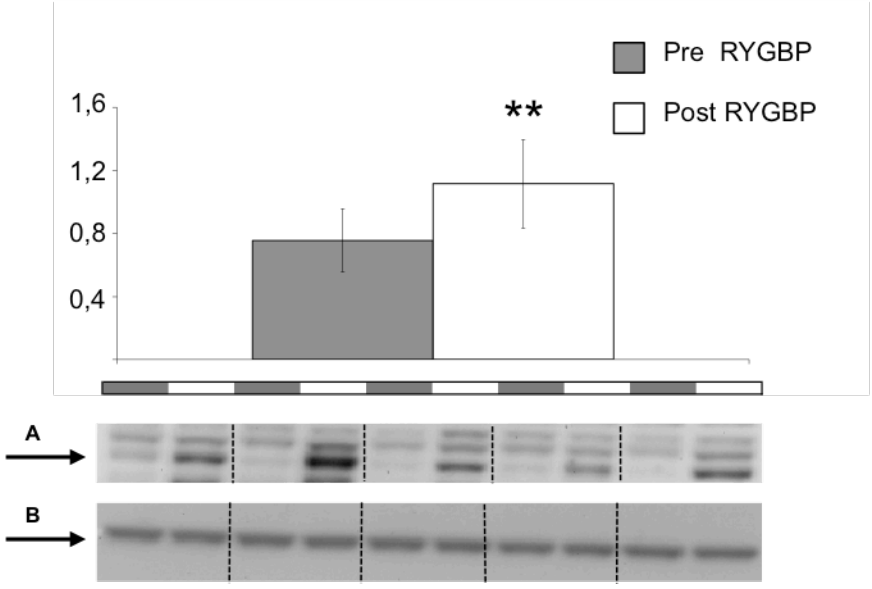
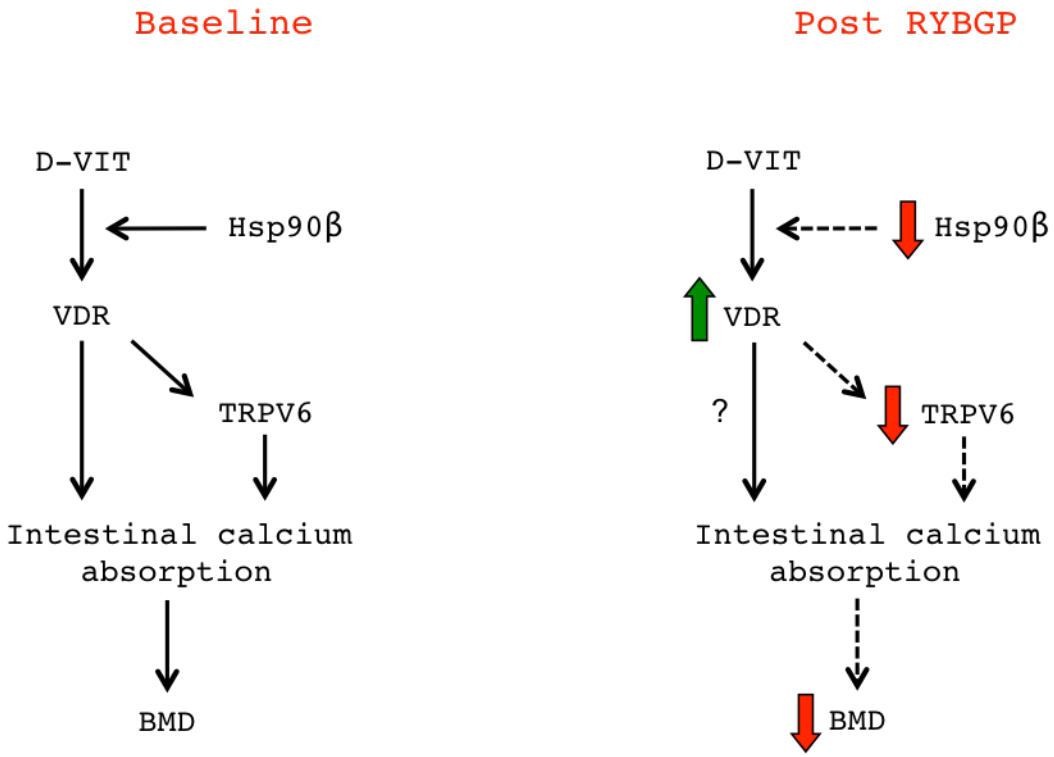


Figure 5 Schematic presentation of presumptive changes of intestinal calcium absorption mechanism after RYGBP



8.2. Brief Report: “Estradiol increases body-weight loss and gut-peptide satiation after Roux-en-Y gastric bypass in ovariectomized rats”

This section contains a brief report that was submitted for publication to Gastroenterology in February 2012 and accepted for publication in revised form in May 2012.

My contribution to this manuscript includes data acquisition, data interpretation and revising the manuscript.

Estradiol Increases Body Weight Loss and Gut-Peptide Satiation After Roux-en-Y Gastric Bypass in Ovariectomized Rats

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Despite the fact that ~85% of bariatric operations are performed in women, the effects of the reproductive axis function on outcome of bariatric surgery remain to be determined. Here we developed the first published model of Roux-en-Y gastric bypass (RYGB) in female rats. We show in ovariectomized rats receiving estradiol or control treatment that (1) RYGB-induced body weight loss and (2) the satiating efficacy of endogenous glucagon-like peptide-1 and cholecystokinin satiation were significantly increased in estradiol-treated rats. These data are relevant to the care of obese women, in particular perimenopausal women, undergoing bariatric surgery.

Keywords: Bariatric Surgery; Obesity; CCK; GLP-1.

Bariatric surgery is currently the most effective treatment for morbid obesity. Morbid obesity (body mass index >40 kg/m^2) is more prevalent in women than in men,[1,2] and the adverse effects of obesity on psychological well-being are more pronounced in women.[3] Correspondingly, more than 85% of patients undergoing bariatric surgery are female.[4] Despite the well-known effects of normal reproductive axis function on eating and body weight,[5] the impact of reproductive axis function on the outcome of bariatric surgery remains to be determined. Here we investigated the effects of Roux-en-Y gastric bypass (RYGB) surgery on body weight and glucagon-like peptide 1 (GLP-1)– and cholecystokinin (CCK)-induced satiation in a rat model of menopause.

Adult Long–Evans female rats were fattened for 3 weeks by offering only a high-energy diet (phase 1) before ovariectomy, the standard rodent menopause model, and RYGB or sham surgery (Supplementary Materials and Methods). Rats were then fed high-energy and regular chow ad libitum for 28 days (phase 2). A near-physiological regimen of estradiol treatment (2 μg estradiol benzoate once each 4th day subcutaneously) or oil-vehicle treatment (100 μL sesame oil) was begun on day 12, resulting in 4 groups: SHAM-E2, SHAM-OIL, RYGB-E2, and RYGB-OIL. Beginning on day 29, rats were fed Ensure Plus (Abbott AG, Baar, Switzerland) liquid diet and chow for 21 days (phase 3). Finally, the acute effects of exendin (9–39) and devazepide on Ensure intake were tested (phase 4).

At arrival, rats weighed 165 ± 6 g. During phase 1, rats gained ~80 g, reaching an overall mean preoperative body weight of 247 ± 3 g (Figure 1*A*). The continued rapid weight gain of SHAM-OIL rats during phases 2 and 3 was significantly attenuated in RYGB-OIL rats (19 vs 42 g and 30 vs 67 g in phases 2 and 3, respectively; standard errors of the difference, 8 and 7 g, respectively; Figure 1*A*). The weight-lowering effect of RYGB was significantly greater in estradiol-treated rats than oil-treated rats by 31 and 49 g in phases 2 and 3, respectively. Overall, SHAM-OIL rats gained 109 g during phases 2 and 3 versus 49 g in RYGB-OIL rats and −1 g in RYGB-E2 rats.

RYGB and estradiol treatment had similar effects on energy intake as on body weight both during phase 2, when rats were fed a solid, high-energy diet, and during phase 3, when the rats were fed Ensure, which increased energy intake in all groups (Figure 1*B*). RYGB-OIL rats ate less than SHAM-OIL rats in both phases, RYGB-E2 rats ate less than RYGB-OIL in phase 2 and tended to eat less in phase 3 ($P < .06$), and SHAM-E2 rats ate less than SHAM-OIL rats in both phases. RYGB also reduced the rats' selection of both test diets versus chow (Table 1).

We tested the effects of exendin (9–39) and devazepide, which are potent and selective receptor antagonists of GLP-1 and CCK, respectively, during 60-minute Ensure tests (phase 4). In both sham-operated and RYGB rats, the eating-stimulatory effects of the antagonists were significantly greater in estradiol-treated than oil-treated rats (Figure 2). In neither case, however, was the antagonist effect greater in RYGB-E2 rats than in SHAM-E2 rats.

We consider three aspects of our data important. First, to our knowledge, this is the first study investigating the influence of reproductive axis function on the outcome of bariatric surgery. These data are critical because the great majority of gastric bypass operations are performed in women and because the reproductive axis, in particular estrogens, potently influence the physiology of eating and body weight control. Second, we showed that estradiol significantly increased the potency of RYGB to reduce weight gain and to inhibit eating. Similar effects were obtained when RYGB rats were fed a high-energy solid diet (phase 2) and Ensure, a 57% energy liquid diet (phase 3), suggesting that they do not depend on particular dietary forms or components. Our data suggest that RYGB may be more effective in healthy premenopausal

Abbreviations used in this paper: GLP-1, glucagon-like peptide 1; RYGB, Roux-en-Y gastric bypass.

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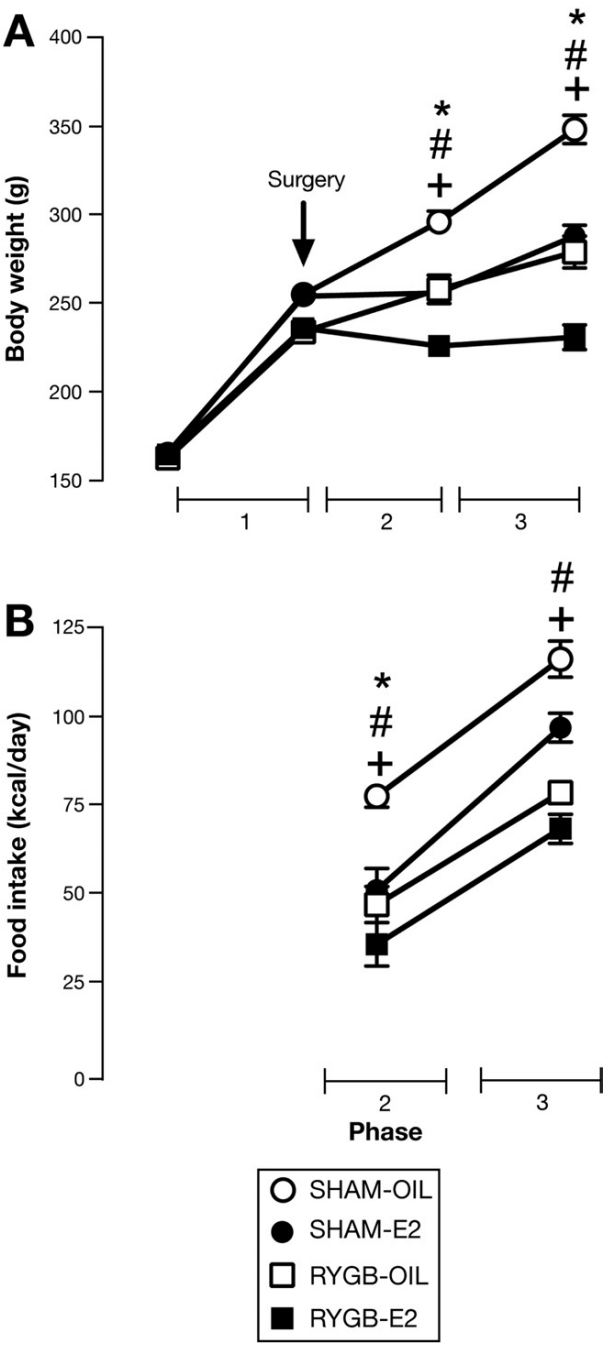

Figure 1. (*A*) Body weight and (*B*) food intake following RYGB or sham operation in ovariectomized rats that were treated with estradiol (E2) or the oil vehicle (OIL). #SHAM-OIL vs RYGB-OIL; *RYGB-OIL vs RYGB-E2; +SHAM-OIL vs SHAM-E2; $P < .01$ for body weight gains and $P < .05$ for food intake.

women than in postmenopausal women who do not receive hormone replacement therapy or in women with reduced estrogen levels, such as patients with polycystic ovary syndrome.[6] We are not aware of any clinical studies assessing this question, although we found recently that bariatric surgery was more effective in postmenopausal-age women than premenopausal-age women (Ochner,

Table 1. Percent of Each Diet Consumed Out of the Overall Intake

	Phase 2		Phase 3	
Group	HFD	CHOW	ENSURE	CHOW
SHAM-OIL	71 ± 7	29 ± 7	99 ± 0.3	1 ± 0.3
SHAM-E2	74 ± 4	26 ± 4	98 ± 0.3	2 ± 0.3*
RYGB-OIL	52 ± 11	48 ± 10	92 ± 3	8 ± 4+
RYGB-E2	52 ± 11	48 ± 10	77 ± 5+	23 ± 4*+

*vs OIL within diet and surgical group.
+vs SHAM within diet and hormone group.

Geary, and Asarian, unpublished data, September 2007). Whether estrogens enhance the eating and body weight–lowering effects of RYGB via the same mechanisms by which they contribute to the normal control of eating and body weight also deserves further research. Furthermore, it is unclear whether estrogens affect the outcome of other types of bariatric surgery procedures, such as gastric banding or gastric sleeve resection. Third, we provide novel evidence that estrogens increase endogenous GLP-1 and

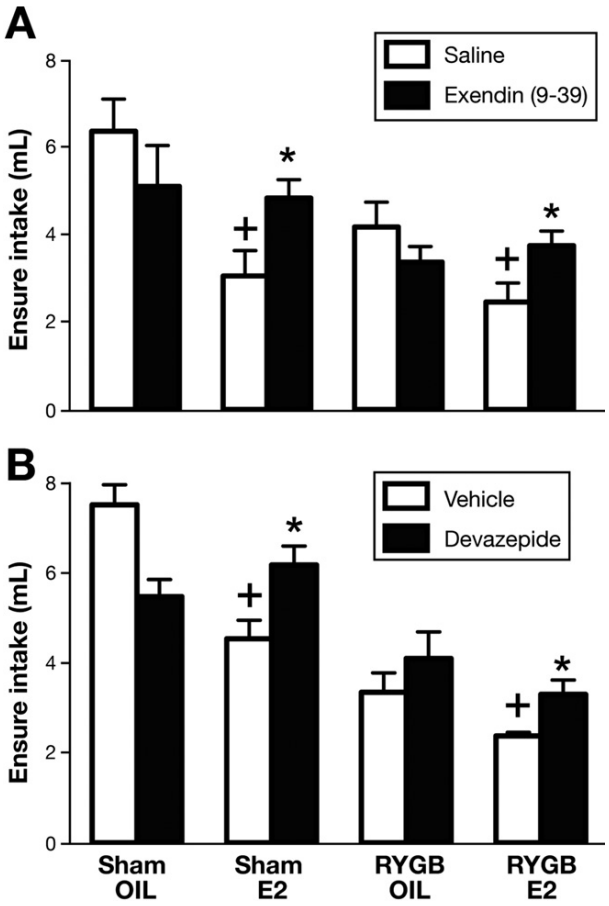

Figure 2. Effects of RYGB on the de-satiating actions of (*A*) GLP-1 antagonism with exendin (9–39) and (*B*) CCK antagonism with devazepide in ovariectomized rats treated with estradiol (E2) or the oil vehicle (OIL). +Estradiol decreased Ensure intake after control injections in the same surgery group; *antagonist increased Ensure intake in the same surgery group; $P < .05$.

CCK satiation in RYGB rats. In previous reports, estradiol increased CCK satiation in rats with intact intestines.[7–9] This is the first report that estradiol also increases GLP-1 satiation in intestine-intact rats and that estradiol increases the satiating effect of CCK and GLP-1 in RYGB rats. We did not, however, detect any difference in the potency of either GLP-1 or CCK antagonism in estradiol-treated RYGB versus estradiol-treated, sham-operated rats, suggesting that increases in the satiating potencies of these peptides alone are not sufficient to account for the decreased total food intake and decreased weight gain in our model of RYGB. Possibly larger doses of the antagonists, chronic antagonist treatment, or modifications of the RYGB method (eg, longer intestinal bypass) would reveal a surgical effect. Finally, other estrogenic effects, such as metabolic effects, may also have contributed.

Supplementary Materials

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi:10.1053/j.gastro.2012.05.008.

References

1. Bray GA. J Clin Endocrinol Metab 2004;89:2583–2589.
2. Flegal KM, et al. JAMA 2010;303:235–241.
3. Carpenter KM, et al. Am J Public Health 2000;90:251–257.
4. Samuel I, et al. Am J Surg 2006;192:657–662.
5. Asarian L, et al. Philos Trans R Soc Lond B Biol Sci 2006;361:1251–1263.
6. Escobar-Morreale HF. J Clin Endocrinol Metab 2005;90:6364–6369.
7. Asarian L, et al. Peptides 1999;20:445–450.
8. Asarian L, et al. Endocrinology 2007;148:5656–5666.
9. Eckel LA, et al. Peptides 1999;20:451–456.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Subjects and Housing

Adult, female Long–Evans rats (Centre d'Elevage R. Janvier, Le Genest-Saint-Isle, France) weighing ~165 g at arrival were used. Rats were initially housed 4 per cage in Makrolon cages (56 × 38 × 22 cm) with wood chip bedding (Abbed Espen, Indulab AG, Holland). Postsurgery rats were single housed in stainless-steel-wire-mesh cages (48 × 25 × 18 cm). Rats were housed in a colony room with an average temperature of 23 ± 2°C and a 12-hour light/dark cycle (lights off at 1300). The Cantonal Zurich Veterinary Office approved all procedures.

Materials

17β-estradiol-3-benzoate (catalog no. E8515; Sigma, Schnelldorf, Germany), sesame oil (catalog no. S3547; Sigma), exendin (9–39) (catalog no. H8740; Bachem, Bubendorf, Switzerland), devazepide (catalog no. 2304; Tocris Biosciences, Bristol, England), purified condensed-milk high-energy diet (D12266B, 4.41 kcal/g, 25% energy from sugar, 32% from fat; Research Diets, New Brunswick, NJ), Ensure Plus Chocolate (catalog no. S256, 1.5 kcal/mL, 57% energy from sugar, 28% from fat; Abbott AG, Baar, Switzerland), laboratory chow (catalog no. 3430, 3.15 kcal/g, 41% energy from carbohydrates, 4.5% from fat; Provimi Kliba, Kaiseraugst, Switzerland).

Methods

Surgeries. Forty-eight rats were allocated to either RYGB (n = 24, body weight, mean ± SD, 237 ± 10 g) or sham-operated (sham, n = 24, 237 ± 15 g) surgery groups. Anesthesia was induced in a chamber filled with 5% isoflurane in oxygen (1 L/min). After an adequate depth of anesthesia was achieved, each rat was shaved from sternum to pelvis followed by disinfection with Betadine scrub (Mundi Pharma, Basel, Switzerland). The rat was then placed in a supine position on a heating pad and positioned in a nose cone to maintain anesthesia (2%–4% isoflurane in oxygen, 0.5 L/min) for the duration of the surgery. All surgeries were conducted as previously described.[1] Briefly, a midline incision of approximately 4 cm starting just below the xiphoid process was performed. For the RYGB procedure, the small bowel was transected approximately 20 cm distal to the pylorus of the stomach, creating a proximal and distal end of small bowel. The proximal end, being still continuous with the remaining portion of the stomach, constituted the biliopancreatic limb and was anastomosed to the ileum approximately 25–30 cm from the cecum, creating the common channel. For formation of the gastric pouch, the stomach was transected approximately 5 mm below the gastroesophageal junction, creating a gastric pouch of a size of no more than 2%–3% of original stomach size. The Roux-en-Y reconstruction was completed by connecting the distal end of the small bowel to the gastric pouch, leading to formation of the alimentary limb. One single RYGB procedure lasted approximately 70 minutes. For sham operations, an anterior gastrostomy and a jejunostomy with subsequent closures were performed. One single sham procedure lasted approximately 30 minutes. The abdominal wall and the skin were closed in layers after both operations.

Bilateral ovariectomy was performed subsequent to RYGB or sham surgery. A suture was placed between the ovary and the tip of the uterus, and the ovary was cut free. The ovary was then separated from the retroperitoneal fat with a cautery and removed. Postoperatively, enrofloxacin (0.15 mg/kg; Baytril, Bayer, Provet AG, Lyssach, Switzerland), flunixin meglumine (5 mg/kg; Biokema SA, Lausanne, Switzerland), and meloxicam (5 mg/kg; Boehringer Ingelheim, Basel, Switzerland) were administered for infection and pain prophylaxis, respectively. Two RYGB rats died during surgery. Immediately following surgery, each rat received 5 mL of warm saline subcutaneously to compensate for fluid loss. Rats were then placed under indirect red light in a polycarbonate cage until they fully recovered from anesthesia, at which time they were returned to their home cages. Baytril was also administered on the first postoperative day. Rats were allowed at least 10 days to recover before further testing. During recovery, rats were offered chow, high-energy diet, water, and, for RYGB rats, 5% sucrose solution during the first week. Recovery was uneventful for most rats, but 7 RYGB rats became acutely ill 3–7 days postoperatively and either died or were killed with a pentobarbital overdose; necropsy indicated that each of these rats had severe peritonitis secondary to failed intestinal anastomoses.

Hormone replacement treatment. Cyclic estradiol treatment was begun after 12 days of postoperative recovery. RYGB rats were divided into 2 groups of approximately equal body weight and received, every fourth day, single interscapular subcutaneous injections of 2 μg estradiol in 100 μL sesame oil or oil alone (RYGB-E2, n= 7; RYGB-OIL, n = 8). Sham rats were culled to similar group sizes and similarly allocated (SHAM-E2, n = 8; SHAM-OIL, n = 9). Injections were performed between 0900 and 0930 h. This regimen produces increases in plasma estradiol levels similar in magnitude and duration to those during 4-day ovarian cycles in intact rats.[2,3]

Test procedure. The experiment consisted of 4 phases. Surgery was performed on day 0. In phase 1 (days −21 to −1), beginning 5 days after the rats arrived, rats were fed the high-energy diet to induce overweight and to mimic human idiopathic "dietary" obesity.

In phase 2 (days 0 to 28), estradiol or oil treatment was begun on day 12. Rats were offered high-energy diet and regular chow ad libitum throughout. Food intake and body weight were measured daily between 0900 and 0930.

During phase 3 (days 29 to 50) rats were offered Ensure and chow ad libitum; the method was otherwise unchanged.

During phase 4 (days 51 to 69), the satiating potencies of endogenous GLP-1 and CCK were tested in within-subject crossover tests, performed during the second nocturnal period after estradiol or oil injections, when, similar to estrus in intact, cycling rats, the effect of estradiol on eating is greatest.[2]

The satiating potency of endogenous GLP-1 was tested with the GLP-1 receptor antagonist exendin (9–39) using a method similar to that of Williams et al.[4] Rats were deprived of both chow and Ensure, but not water, 4 hours before dark onset (0900). At dark onset (1300), Ensure was returned. At 1400, intake of Ensure was measured and 100 μg/kg exendin (9–39) or the saline vehicle (1 mL/kg) was injected intraperitoneally. Intake of Ensure was measured 1 hour later (1500). Finally, Ensure and chow were provided ad libitum until the next day at 0900.

The satiating potency of endogenous CCK was tested with the CCK-1 receptor antagonist devazepide using a method similar to that used previously.[5] Rats were deprived of food at 0900, 4 hours before dark onset. At 1200, 1 mg/kg devazepide or vehicle (1:10 dimethyl sulfoxide/saline, 1 mL/kg) was intraperitoneally injected. Ensure was returned at 1300 (dark onset), and intake was measured 1 hour later (1400). Ensure and chow were then provided ad libitum until the next day at 0900.

Statistical Analyses

The difference in body weight between RYGB and sham rats after phase 1 was ~20 g. This occurred because the RYGB rats that died during and after surgery (n = 9) were a bit heavier than those that did not. Figure 1 shows data for the surviving rats. This difference prompted us to analyze changes in body weight rather than absolute weights during phases 2 and 3. Changes in body weight were analyzed using planned comparisons, with standard errors of the difference (SED) computed from analysis of variance–generated residual errors and Bonferroni–Hochberg corrected *t* tests. Comparisons were (1) whether RYGB decreased weight gain in oil-treated rats (ie, SHAM-OIL vs RYGB-OIL), (2) whether estradiol increased this effect (ie, RYGB-OIL vs RYGB-E2), and (3) whether estradiol decreased weight gain in sham-operated rats (ie, SHAM-OIL vs SHAM-E2) as a positive control for the effect of estradiol in these novel diet conditions; SEDs were 8.3 g in phase 2 and 6.6 g in phase 3. Mean daily intakes of high-energy diet, Ensure, and chow were converted to kilocalories, and intake of total kilocalories per day during the last weeks of phases 2 and 3 were analyzed as previously described; SEDs were 4.1 kcal/day in phase 2 and 6.2 kcal/day in phase 3. The comparisons used to test the effects of exendin (9–39) and devazepide on 1-hour Ensure intakes in phase 4 were (1 and 2) whether estradiol reduced food intake after control injections in sham-operated rats (ie, SHAM-OIL/control vs SHAM-E2/control) or in RYGB rats (ie, RYGB-OIL/control vs RYGB-E2/control), (3 and 4) whether the antagonist increased Ensure intake in estradiol-treated rats (ie, SHAM-E2/control vs SHAM-E2/antagonist and RYGB-E2/control vs RYGB-E2/antagonist), and (5) whether the effects of the antagonist differed between sham-operated and RYGB rats (ie, 3 vs 4). To increase statistical power, the effects of the antagonists were tested using one-tailed tests. SEDs were 0.9 mL for the exendin (9–39) tests and 0.5 mL for the devazepide tests.

Supplementary References

1. Bueter M, et al. Obes Surg 2010;20:616–622.
2. Asarian L, et al. Horm Behav 2002;42:461–471.
3. Micevych P, et al. J Neurobiol 1996;30:465–479.
4. Williams DL, et al. Endocrinology 2009;150:1680–1687.
5. Asarian L, et al. Peptides 1999;20:445–450.

8.3. Brief communication: “Roux-en-Y gastric bypass surgery in rats alters gut microbiota profile along the intestine”

This section contains a brief communication that was submitted for publication to Physiology & Behavior in November 2012 and accepted for publication in revised form in June 2013.

My contribution to this manuscript includes data acquisition and revising the manuscript.

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Brief communication

Roux-en-Y gastric bypass surgery in rats alters gut microbiota profile along the intestine☆

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ABSTRACT

Roux-en-Y gastric bypass (RYGB) surgery might modify the gut microbiota composition differently in the three distinct anatomical sections of the small intestine compared to sham surgery. We showed that RYGB induced changes in the microbiota of the alimentary limb and the common channel resembling those seen after prebiotic treatment or weight loss by dieting. These changes may be associated with altered production of intestinal hormones known to control energy balance. Postsurgical modulation of gut microbiota may significantly contribute to the beneficial metabolic effects of RYGB surgery.

1. Introduction

Recent studies examined the association between changes in intestinal microbial diversity in obese rodents and humans; some bacterial groups were associated with changes in the nutritional status. Obesity was associated with higher *Firmicutes* and lower *Bifidobacterium* spp., *Bacteroides*-related bacteria and *Lactobacillus* spp. in comparison with the lean counterparts [1–4]. Interestingly, weight loss achieved by dieting was able to reverse those changes [5]. Furthermore, nutrients with prebiotic properties induced qualitative changes in the composition of the gastrointestinal microbiota and peptide release (e.g., glucagon-like peptide-1 (GLP-1)) similar to those seen after dieting. In diet-induced obese and type 2 diabetic (T2DM) mice the release of gut peptides induced by treatment with prebiotics improved glucose and lipid metabolism as well as systemic inflammation [6].

Roux-en-Y gastric bypass (RYGB) is currently, the most effective strategy for long term weight loss maintenance. RYGB significantly reduces body weight, improves T2DM and changes the postprandial enteric endocrine responses.

Gut microbiota analysis of fecal samples from humans and rats after RYGB suggested that the reduction of *Firmicutes* and Bacteroidetes may partly explain the weight loss and beneficial effects on metabolism and inflammation associated with the RYGB surgery [7–9]. Liou [10] confirmed these findings and also showed that cecal transplants from mice after RYGB to unoperated germ free mice decreased the body weight and adiposity compared to recipients of microbiota from sham-operated mice.

There are currently no data on the impact of gut microbiota on the hormonal and metabolic changes associated with RYGB. In most of the human and rodent studies investigating the ecology and activity of intestinal microbiota, fecal or cecal samples have been used. However, these may not be representative of the microbiome in RYGB where the intestine is surgically manipulated into three discrete sections which may each contribute to distinct metabolic signals compared to feces that represents a amalgamate of the microbiome from the intestine as a whole. Therefore, we assessed the bacterial composition in the different anatomically corresponding intestinal segments after RYGB or sham surgery.

2. Materials and methods

2.1. Subjects and housing

Sixteen male Wistar rats (Harlan Laboratories Inc., Blackthorn, UK; Elevage Janvier, Le-Genest-St. Isle, France) were individually housed under a 12 h/12 h light–dark cycle at a room temperature of 21 ± 2 °C. Water and standard chow were available ad libitum. All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland. All rats were given one week of acclimatization before

Abbreviation: DPP 4, dipeptidyl peptidase-4.

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being randomized to RYGB (n = 8) or sham-operation (n = 8). After surgery, rats received Ensure (chocolate Ensure Plus, Abbott Nutrition, Baar, Switzerland) for 3 days before access to normal chow was reinstalled. Body weight was measured weekly. A food restricted sham-operated group of rats (n = 7), whose postoperative weight matched the weight of bypass-treated animals, was also studied (data not shown; see also ref [11]); data were comparable to the sham-operated ad libitum fed controls except for one parameter (see below).

2.2. Surgery

Rats were allocated to either RYGB (n = 8, body weight, mean ± SEM, 445 ± 5 g) or sham-operated (sham, n = 8; 435 ± 5 g) surgery groups. Anesthesia was induced in a chamber filled with 5% isoflurane in oxygen (1 L/min). After an adequate depth of anesthesia was achieved, rats were shaved from sternum to pelvis followed by disinfection with Betadine scrub (Mundi Pharma, Basel, Switzerland). Rats were then placed in a supine position on a heating pad and positioned in a nose cone to maintain anesthesia (2%–4% isoflurane in oxygen, 0.5 L/min) for the duration of the surgery. All surgeries were conducted as previously described [11–13] and the small intestinal segments after RYGB (biliopancreatic limb; alimentary limb; common channel) are depicted in Fig. 1. Briefly, a midline incision of approximately 4 cm starting just below the xiphoid process was performed. For the RYGB procedure, the small bowel was transected approximately 20 cm distal to the pylorus of the stomach, creating a proximal and distal end of the small bowel. The proximal end, being still continuous with the remaining portion of the stomach, constituted the biliopancreatic limb and was anastomosed to the ileum approximately 25–30 cm from the cecum, creating the common channel. For formation of the gastric pouch, the stomach was transected approximately 5 mm below the gastroesophageal junction, creating a gastric pouch of a size of no more than 2%–3% of original stomach size. The Roux-en-Y reconstruction was completed by connecting the distal end of the small bowel to the gastric pouch, leading to formation of the alimentary limb. One single RYGB procedure lasted approximately 70 min. For sham operations, an anterior gastrostomy and a jejunostomy with subsequent closures were performed. One single sham procedure lasted approximately 30 min. The abdominal wall and the skin were closed in layers after both operations.

2.3. DNA isolation

The content of intestinal tracts (n = 6–8 per group) collected immediately after euthanasia was stored at −80 °C (biliopancreatic limb, alimentary limb, common channel, cecum and colon after RYGB and in the anatomically corresponding segments after sham surgery).

Metagenomic DNA was extracted from the intestinal content using a QIAamp-DNA stool minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.4. qPCR: primers and conditions

The primers and probes used to detect total bacteria, *Bifidobacterium* spp., *Lactobacillus* spp. and *Bacteroides–Prevotella* spp. were based on 16S rRNA gene sequences and as described by Vincent et al. [14,15]. Detection was achieved with a STEP one PLUS instrument and software (Applied Biosystems, Foster City, CA) using a MESA FAST quantitative PCR MasterMix Plus for SYBR Assay (Eurogentec, Verviers, Belgium). Each assay was performed in duplicate in the same run. The cycle threshold of each sample was then compared to a standard curve (performed in triplicate) made by diluting genomic DNA (five-fold serial dilution) (BCCM/LMG, Ghent, Belgium). The statistical analysis was done on logarithmic values.

2.5. DDP IV activity

Snap frozen intestinal tissue samples (200 mg) were homogenized in 1 mL cold homogenization buffer (10 mM Tris–HCl, pH 8.2), and analyzed immediately for enzyme activity. The serum from blood samples was separated by centrifugation and stored at −20 °C until analysis of enzyme activity.

Determination of intestinal and serum dipeptidyl peptidase-IV (DPP IV) activities, which degrades gut peptides like GLP-1, was performed as described by Kreisel et al. [16]. DPP IV activities were determined by measuring the release of 4-nitroaniline from an assay mixture containing 0.1 mol Tris–HCl (pH 8.0), 2 mmol Gly-Pro

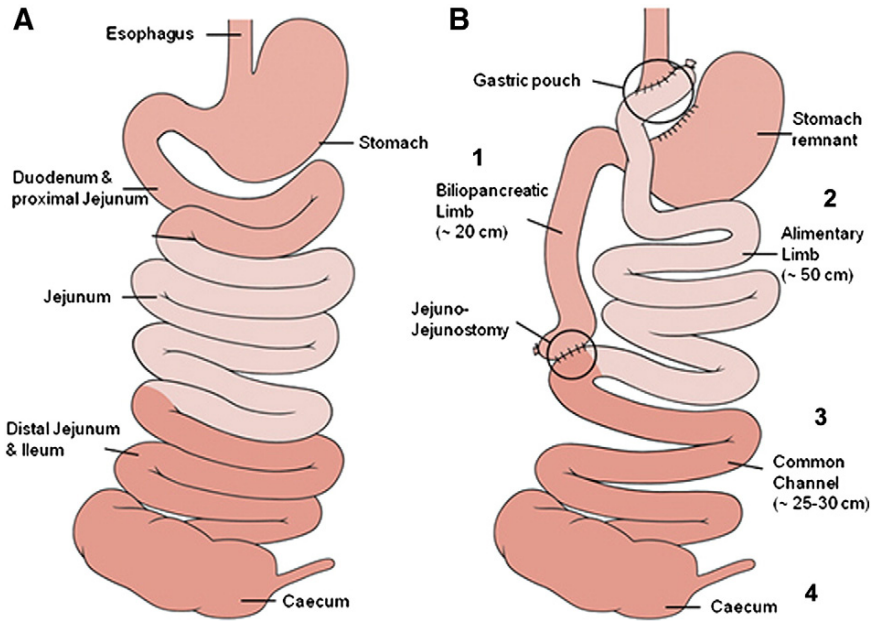

Fig. 1. Schematic illustration of the surgical diagram of our animal model of RYGB. A: Normal gut anatomy. B: Gut anatomy after RYGB surgery: 1: biliopancreatic limb/duodenum. 2: alimentary limb/proximal jejunum. 3: common channel/distal jejunum. 4: cecum.

p-nitroanilide (Sigma-Aldrich, Saint Louis MO, USA) as the substrate and enzyme in a total volume of 0.20 mL. After 30 min of incubation at 37 °C, the reaction was stopped by the addition of 0.4 mL of 2 M sodium acetate buffer (pH 4.5). Human recombinant DPP IV (Sigma-Aldrich, Saint Louis MO, USA) was used as standard. The absorbance at 405 nm was measured by the use of a Lab Systems Multiskan RC 96-well plate reader (Thermo Fisher Scientific, Waltham MA, USA). All reactions were performed in duplicate.

Protein concentrations in homogenates were determined according to the method of Pierce [17]. Enzyme activities in homogenates were expressed as international units per gram of protein, and in serum as international units per liter of serum. One unit corresponds to the hydrolysis of 1 mmol of substrate per minute under the assay conditions.

2.6. Statistical analyses

Results are presented as mean ± S.E.M. Statistical significance of difference between groups was assessed by Mann–Whitney nonparametric t-test (Graph-Pad Prism Software, San Diego, CA, USA; www.graphpad.com).

3. Results and discussion

Average presurgical body weight of rats was 430 ± 4 g. Seven days after surgery, sham-operated controls weighed significantly more compared with gastric bypass rats (sham: 370 ± 9 g vs. bypass: 450 ± 6 g, $p < 0.001$). Body weight changes for both groups are shown in Fig. 2.

Total bacterial content was significantly increased in the alimentary limb and common channel after RYGB compared to sham rats. In the cecum after RYGB the changes in the microbial ecology were similar to that seen after prebiotic treatment [4], with *Bifidobacterium* spp. and *Lactobacillus* spp. and were significantly lower. RYGB also increased *Bifidobacterium* spp. and *Bacteroides–Prevotella* spp. in the common channel, the alimentary limb and in the colon (Fig. 3). After RYGB surgery, DPP IV activity was decreased by 27% in the alimentary limb ($p = 0.01$) and by 32% in serum ($p = 0.002$) (Fig. 4).

Most changes in microbial composition seen in the RYGB rats were body weight-independent. In fact, sham-operated body weight-matched rats did not show significant modification of gut microbiota compared to ad libitum fed sham-operated controls, with the exception for the increase in *Bacteroides Prevotella* spp. in the alimentary limb and the common channel of the body-weight matched rats (data not shown). Although changes in gut microbiota seen after RYGB resembled those obtained after weight loss in obese rodents [5], our data indicate that RYGB may have specific effects on intestinal microbiota.

The major finding of the study is that the most substantial shifts in the composition of the microbiota were observed in the alimentary limb and the common channel. Interestingly, we recently found that the total gene expression of the gut hormones preproglucagon, peptide YY and cholecystokinin was increased in the alimentary limb and common channel of RYGB rats but not in sham-operated rats that were body weight-matched to the RYGB rats [18].

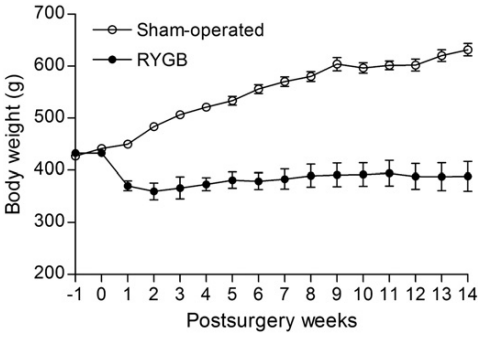

Fig. 2. Body weight change for the gastric bypass (-●-) (n = 8) and sham-operated rats ad libitum fed (-○-) (n = 8). Data are expressed as mean ± SD.

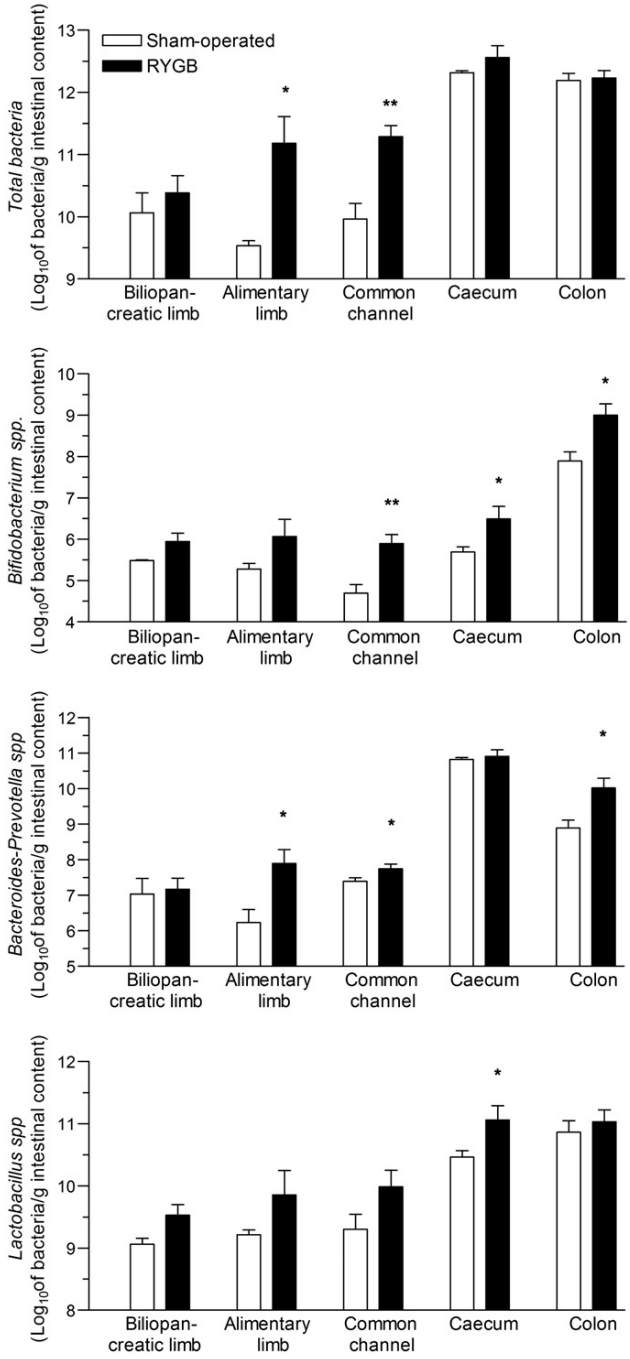

Fig. 3. RYGB-associated changes in gut microbiota. Results are given as the Log_{10} of bacterial/intestinal content (g). Data are expressed as mean ± S.E.M $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to sham-operated rats.

Our findings suggest that the bypass of the proximal intestine may contribute to the changes of the gut microbiota observed after RYGB. The exclusion of the proximal intestine from contact with ingested nutrients has been demonstrated to play a major role in the beneficial effects of RYGB surgery [19]. Although a putative mechanism promoting such beneficial effects of the surgery remains to be elucidated, our data suggest a possible role for changes in the microbial composition of the proximal small bowel.

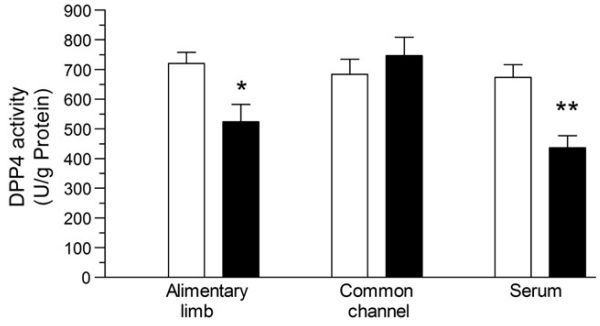

Fig. 4. RYGB-associated changes in DPP 4 activity. Data are expressed as mean ± S.E.M *p < 0.05; **p < 0.01 compared to sham-operated rats.

In the present study, a decreased activity of intestinal and serum DDP IV activity was detected. A direct link between the decreased DPP IV activity and changes in gut microbiota composition was not investigated; however, we have previously shown that prebiotic-induced changes in gut microbiota and increased GLP-1 levels were associated with reduced DPP IV activity [20]. The gut microbiota changes that we observed in this study resemble in part those seen after treatment with prebiotics, thus we think that similar mechanisms may have prevailed under the conditions of our study.

The modest decrease in serum DPP IV activity in response to RYGB compared to pharmacological approaches is consistent with other studies in humans [21]. It could be secondary to the experimental conditions; it is e.g. known that the diabetic status may influence the level of DPP4 activity [22]. In our RYGB model, however, the difference of glucose tolerance between sham-operated and RYGB is not supposed to differ as much as in some models of obesity and type 2 diabetes frequently used to test pharmacological approaches. Hence, we expected the difference of DPP-4 activity in our study to be smaller.

Further, because DPP-4 is mainly a tissue enzyme, changes in serum DPP-4 activity may not completely reflect the total decrease in activity of the enzyme and the consequent decreased inactivation of incretins. In the present study, DPP-4 activity in the alimentary limb was also decreased and might explain the robust increase in circulating incretin levels usually detected in our RYGB rat model [1]. In other words, reduced intestinal and serum DDP IV activity may contribute to enhanced bioavailability of endogenous GLP-1 and be consistent with the modulation of jejunal microbiota altering the production or breakdown of gastrointestinal hormones known to control energy balance.

4. Conclusions

In conclusion, these data are critical because they accurately represent how RYGB surgery may affect microbial communities in different intestinal segments after surgery. Because most changes in gut microbiota were independent from weight loss, we conclude that other mechanisms than weight loss per se seem to be responsible for the alteration of the intestinal microbial population after RYGB surgery.

Postsurgical modulations of the gastrointestinal microbial community, e.g. the bypass of the intestinal foregut, may influence gut peptide synthesis, release and breakdown; hence, these changes may significantly contribute to the beneficial metabolic effects of RYGB surgery independent of the RYGB-induced body weight loss. The transplant experiment with cecal contents of RYGB mice clearly is consistent with this idea [10].

The impact of RYGB on the microbiota of the different intestinal tracts remains to be confirmed in other experimental models, e.g. in a high fat diet or a genetic model of obesity, and in large-scale studies including body weight-matched controls and using pyrosequencing, metagenomic analysis and metabolic profiling. These tools will enable further insight into the composition of the intestinal microbiota and its functional evolution after bariatric surgery. Elucidating the mechanisms by which gut microbiota interact with the host will provide a new basis for putative pharmacological or dietary intervention for obesity and its related comorbidities.

Disclosure

The authors declare no conflict of interests.

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Author contribution

MO; TAL: study concept and design;
MO; KA; MB: acquisition of data;
MO; PDC; TAL: analysis and interpretation of data;
MO; TAL: drafting of the manuscript.
MB; CWR; PDC; TAL: critical revision of the manuscript for important intellectual content.

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References

[1] Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444:1022–3.
[2] Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457:480–4.
[3] Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31.
[4] Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of *Bifidobacteria* in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia 2007;50:2374–83.
[5] Duncan SH, Lobley GE, Holtrop G, Ince J, Johnstone AM, Louis P, et al. Human colonic microbiota associated with diet, obesity and weight loss. Int J Obes 2008;32:1720–4.
[6] Everard A, Lazarevic V, Derrien M, Girard M, Muccioli GG, Neyrinck AM, et al. Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes 2011;60:2775–86.
[7] Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci U S A 2009;106:2365–70.
[8] Furet JP, Kong LC, Tap J, Poitou C, Basdevant A, Bouillot JL, et al. Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. Diabetes 2010;59:3049–57.
[9] Li JV, Ashrafian H, Bueter M, Kinross J, Sands C, le Roux CW, et al. Metabolic surgery profoundly influences gut microbial-host metabolic cross-talk. Gut 2011;60(9):1214–23.
[10] Liou AP, Paziuk M, Luevano JM Jr, Machineni S, Turnbaugh PJ, Kaplan LM. Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. Sci Transl Med 2013;5(178):178ra41.
[11] Bueter M, Lowenstein C, Olbers T, Wang M, Cluny NL, Bloom SR, et al. Gastric bypass increases energy expenditure in rats. Gastroenterology 2010;138:1845–53.
[12] Bueter M, Abegg K, Seyfried F, Lutz TA, le Roux CW. Roux-en-Y gastric bypass operation in rats. J Vis Exp 2012:e3940.
[13] Asarian L, Abegg K, Geary N, Schiesser M, Lutz TA, Bueter M. Estradiol increases body weight loss and gut-peptide satiation after Roux-en-Y gastric bypass in ovariectomized rats. Gastroenterology 2012;143:325–7 [e2].
[14] Dewulf EM, Cani PD, Neyrinck AM, Possemiers S, Holle AV, Muccioli GG, et al. Inulin-type fructans with prebiotic properties counteract GPR43 overexpression and PPARgamma-related adipogenesis in the white adipose tissue of high-fat diet-fed mice. J Nutr Biochem 2010;22:712–22.

[15] Vincent M, Philippe E, Everard A, Kassis N, Rouch C, Denom J, et al. Dietary supplementation with *Agaricus blazei* Murill extract prevents diet-induced obesity and insulin resistance in rats. Obesity (Silver Spring) 2012. http://dx.doi.org/10.1038/oby.2012.139.
[16] Kreisel W, Heussner R, Volk B, Buchsel R, Reutter W, Gerok W. Identification of the 110000 Mr glycoprotein isolated from rat liver plasma membrane as dipeptidylaminopeptidase IV. FEBS Lett 1982;147:85–8.
[17] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85.
[18] Hansen F. L-cell distribution in the GI-tract of ZDF rats. Appetite 2012;Volume 59: e1-61.
[19] Rubino F, Forgione A, Cummings DE, Vix M, Gnuli D, Mingrone G, et al. The mechanism of diabetes control after gastrointestinal bypass surgery reveals a role of the proximal small intestine in the pathophysiology of type 2 diabetes. Ann Surg 2006;244:741–9.
[20] Cani PD, Neyrinck AM, Maton N, Delzenne NM. Oligofructose promotes satiety in rats fed a high-fat diet: involvement of glucagon-like peptide-1. Obes Res 2005;13:1000–7.
[21] Alam ML, Van der Schueren BJ, Ahren B, Wang GC, Swerdlow NJ, Arias S, et al. Gastric bypass surgery, but not caloric restriction, decreases dipeptidyl peptidase-4 activity in obese patients with type 2 diabetes. Diabetes Obes Metab 2011;13:378–81.
[22] Strissel KJ, Stancheva Z, Miyoshi H, Perfield II JW, DeFuria J, Jick Z, et al. Adipocyte death, adipose tissue remodeling, and obesity complications. Diabetes 2007;56: 2910–8.

9. Discussion

Since the individual studies are discussed in detail in the respective articles or sections, I will now only briefly summarize the results and focus on potential links between the studies and try to draw general conclusions from the entire set of experiments.

We showed that RYGB surgery caused weight loss-independent changes in bone metabolism that led to a decrease in BMD, bone volume and trabecular thickness. Bone loss occurred early after surgery and coincided with intestinal calcium malabsorption. However, in contrast to previous reports,[237-242] this seemed to be independent of vitamin D malabsorption, since increased vitamin D activation compensated for lower total vitamin D levels, and PTH levels were not elevated in RYGB rats. Although intestinal calcium absorption normalized between two and seven weeks after surgery, there was no restoration of bone mass. We identified chronic metabolic acidosis, which was associated with increased lactate levels, as a potential mechanism underlying inhibited bone mass restoration. A decrease in BMD was also shown in human patients after RYGB, but not after vertical banded gastroplasty. Increased markers of bone resorption were found, but there was no evidence of secondary hyperparathyroidism or vitamin D deficiency.

We were also able to reproduce the alterations in EE of RYGB rats that we previously reported,[124] and showed that these changes were not an artifact caused by changes in body composition or a shift in the TNZ. However, we could not confirm our hypothesis that increased GLP-1 levels in RYGB rats are directly involved in altering EE, since there was no effect of acute GLP-1 agonism or antagonism on EE.

Finally, we found that weight loss and the satiating effect of CCK and GLP-1 were increased by estrogen replacement in ovariectomized rats, suggesting that RYGB may be more effective in pre- versus postmenopausal women; and provided further evidence supporting recent findings of altered gut microbiota in rats and humans after RYGB.[255-258]

9.1. Effects of RYGB on bone metabolism

9.1.1. Transferability to human RYGB patients

The main findings of our study investigating RYGB-induced changes in bone metabolism in rats were that (1) bone loss occurred independent of body weight loss, (2) calcium malabsorption was only transient, (3) vitamin D activation was increased, thereby compensating for lower total vitamin D levels that were probably caused by intestinal vitamin D malabsorption, and (4) metabolic acidosis associated with increased lactate levels interfered with the regulation of renal calcium homeostasis and potentially inhibited bone mass restoration (see 5).

These results were partly supported by a prospective randomized trial in human patients undergoing either RYGB or vertical banded gastroplasty (see 8.1). The study showed that, although similar weight loss was achieved in both groups, only RYGB led to a marked decrease in both total body and skull BMD. The simultaneous assessment of total body and skull BMD is one strength of the study, because the skull is not a weight-bearing bone. Hence, the decrease in skull BMD after RYGB further supports that bone loss does not occur due to a reduction in mechanical loading of the skeleton. That bone loss is specific to the RYGB procedure and not a general side effect of bariatric surgery is also confirmed in a recent study by Stemmer et al.,[259] which showed similar bone loss in RYGB rats as observed in our study, while there were no significant changes in bone mass in a rat model of vertical sleeve gastrectomy.

Unfortunately, the role of calcium malabsorption could not be determined in the human study since fecal calcium loss was not measured and calcium intake was only evaluated based on a questionnaire. We could, however, confirm the finding of increased active vitamin D levels in human RYGB patients 18 months post surgery, even though they were associated with increased levels of total vitamin D due to postoperative supplementation and therefore with an unaltered active to total vitamin D ratio. Since active vitamin D levels were markedly above the reference range, one may still speculate that the negative feedback of active vitamin D on the vitamin D activating 1α-hydroxylase is attenuated either by a higher requirement for active vitamin D or by other factors directly influencing the feedback mechanism.

Because metabolic acidosis after RYGB has never been reported before, this was not considered in the human study, which commenced long before we performed our experiments. In order to include this factor in future human studies, it is important to further elucidate the origin of increased lactate levels and the contribution of chronic metabolic acidosis to bone loss observed in our rat model. Interestingly, Stemmer et al. reported a decreased pH in the jejunum of RYGB rats,[259] which could be indicative of increased intestinal lactate production. We did not determine the cause of increased lactate levels in our study; however, there are two main hypotheses that could explain an intestinal origin. First, Saeidi et al.[250] recently showed that the gut hypertrophy in RYGB rats was associated with a reprogramming of intestinal glucose metabolism, which led to increased lactate production. Second, the alterations in the intestinal bacterial flora that have repeatedly been described in humans and rats after RYGB surgery[255-258] and that we also demonstrated in our rat model (see 0) could lead to an increase in lactate producing bacteria. It has furthermore been speculated that the gut microbiota can directly regulate bone mass, potentially by interactions with the immune system.[260] Germ-free mice exhibited increased bone mass, which could be normalized by colonalization with normal gut microbiota. The general increase in intestinal bacteria number after RYGB could therefore have a negative impact on bone mass via similar mechanisms. Of note, while the changes in gut microbiota seem to be very similar in rats and humans, however, it has not been

investigated yet if RYGB in humans also leads to gut hypertrophy and altered intestinal glucose metabolism.

9.1.2. Relevance to female patients

We have shown that the effects of RYGB on food intake and body weight loss in ovariectomized female rats are enhanced by an estrogen replacement regimen.[261] This suggests an interaction between estrogen and the factors leading to RYGB-induced weight loss, such as increased gut hormone levels or the action of these hormones, respectively. Since the major influence of menopausal state on bone metabolism is well known,[262,263] it will be of high interest to determine whether there is a similar interaction between estrogen and RYGB-induced bone loss. There are very limited studies on changes in bone metabolism after RYGB surgery that take into account whether the included women were pre- or postmenopausal, and only few studies specifically analyzed differences based on menopausal state.[264,265] However, the scarce literature suggests that postmenopausal women may be more affected by postsurgical changes in bone metabolism.[235,236,264,265] Whether this is caused by a direct interaction between the factors leading to bone loss after RYGB and the effects of decreased estrogen levels or whether it only represents the higher osteoporosis incidence in postmenopausal women in general is unclear. Estrogen exerts its protective effect on bone mass by a complex interaction of several factors. Its most dominant influence is the decrease of bone resorption through suppression of osteoclast formation and activation.[266,267] By inhibiting osteoblast apoptosis,[268,269] it also has direct effects on bone formation. Further, there are several mechanisms by which estrogen affects bone metabolism indirectly, such as an increased activation of vitamin D that leads to higher intestinal calcium absorption.[270] This is of particular interest in the context of our data suggesting a dysregulation of vitamin D metabolism potentially associated with tissue specific alterations in vitamin D signaling in rats.

9.1.3. Future perspectives

Although we were able to show that RYGB-induced bone loss is not simply caused by vitamin D deficiency but is associated with metabolic acidosis and changes in vitamin D metabolism, we were not able to identify the exact mechanisms that led to the decrease in bone mass. One important next step would be to determine whether and how correction of the chronic acidosis may influence the effects of RYGB on bone metabolism in rats, and whether lactic acidosis also occurs in human patients after RYGB.

However, one major challenge when evaluating bone metabolism after RYGB is that the surgery causes changes in various factors interfering with bone homeostasis, and it is extremely difficult to identify the contribution of each factor individually to the observed alterations. The anatomical rearrangement of the gastrointestinal tract has direct effects on calcium and vitamin D absorption, but it may also have indirect effects due to changed neuronal signaling from the intestine. The

adiposity signals insulin and leptin can both affect bone mass negatively by increasing SNS activity,[55,56] since activation of β2-adrenergic receptors on osteoblasts seems to inhibit bone formation and indirectly increase bone resorption.[271] Postprandial insulin secretion is increased after RYGB[115,145] and this potentially coincides with increased postprandial SNS activity.[272] Leptin levels decrease in response to RYGB-induced weight loss, but increased leptin sensitivity could in theory lead to a higher impact of leptin on bone mass after RYGB.[273] Finally, many of the gut hormones that are changed in RYGB patients have recently been shown to be involved in bone mass regulation. Most of them seem to have beneficial effects on bone mass, such as amylin,[274-276] GLP-1,[277] and glucose-dependent insulinotropic hormone,[278] which argues against a role of these hormones in RYGB-induced bone loss. In contrast, PYY may negatively influence bone mass by decreasing osteoblast activity.[279] These findings are however relatively new and there are often contradictory results when comparing in vitro and in vivo studies[280] or central versus peripheral action of such hormones. The concept of the skeleton as an endocrine organ that interacts with the homeostatic control of EE currently receives a lot of attention and is constantly being adapted due to new findings. The RYGB model provides an interesting opportunity to further investigate the connection between bone and energy homeostasis.

9.2. Effects of RYGB on energy expenditure

One main goal of this thesis was to investigate the mechanisms that underlie alterations in EE after RYGB surgery.[124,125] Importantly, we were able to show that these changes were not caused by differences in body composition, i.e. increased lean mass compared to BWM rats, or by a shift in the TNZ leading to increased thermogenesis in RYGB rats at lower temperatures. On the contrary, while the difference in EE between RYGB and BWM rats remained constant at thermoneutrality, the difference between RYGB and AL rats was diminished. This suggests that RYGB rats in fact require less adaptive thermogenesis than AL rats below thermonetrality.

Various factors that have been shown to influence EE are altered by RYGB surgery, including gut hormone levels,[113-115] bile acids,[281-285] meal patterns[118,286] and gut microbiota[255-258,287], and the potential interactions between these factors are therefore manifold. We hypothesized that the marked increase in postprandial GLP-1 secretion after RYGB increases EE via the SNS,[57] but failed to provide evidence to support this idea (see 0). However, since we generally did not find any effect of acute GLP-1 agonism or antagonism on EE in our study although there is evidence for an influence of GLP-1 signaling on EE,[288,289] the negative outcome could potentially be due to the study design. Williams et al.[7] have shown that the effects of GLP-1 antagonism on food intake strongly depend on the study design, i.e. the feeding state of rats and the time point of the light cycle. They further showed that peripheral and central GLP-1 effects may be mediated independently of each other, and it has been suggested that in contrast to peripheral GLP-1 acting as a short-term satiation

signal, central GLP-1 signaling may be involved in long-term control of energy balance.[290] Central GLP-1 infusion has indeed been shown to increase EE compared to pair-fed control mice.[291] Central administration of a GLP-1-antagonist could therefore possibly reveal a role for GLP-1 in EE changes after RYGB. However, in contrast to peripheral GLP-1, alterations in central GLP-1 signaling have not been investigated after RYGB. It is further possible that the subcutaneous injections in our did not lead to the expected results due to a lack of GLP-1 receptor activation on vagal nerve endings, which could have been achieved by intraperitoneal injections. The anorectic dose of intraperitoneally administered GLP-1 agonists is markedly increased in rats after complete subdiaphragmatic vagal deafferentation, suggesting that the effects of peripheral GLP-1 on food intake strongly depend on vagal signaling[292]. If this were also the case for potential effects of peripheral GLP-1 on EE, we could perhaps have detected such effects by intraperitoneal injections. It has to be mentioned though that we did find an effect of both GLP-1 agonism and antagonism on food intake in our rats, and this effect was stronger in RYGB compared to AL rats. This proves that the negative outcome regarding an effect on EE was not caused by a general inefficacy of the drugs or the used doses, but rather suggests that the contribution of GLP-1 or the mediating pathways are different for EE and food intake.

However, in addition to GLP-1, there is also evidence of an involvement of other gut hormones in EE control, and the observed changes in global EE may represent a combined effect of increased levels of all these peptides. This would explain why the antagonism of one single hormone did not lead to detectable changes. Recently, the increase in both fasting and postprandial levels of bile acids after RYGB[281-285] received a lot of attention since bile acids are thought to contribute to improved glucose homeostasis after bariatric surgery.[282-284] They have further been shown to increase EE by BAT stimulation[293-295] and could therefore also play a role in EE control after RYGB.

It has to be mentioned in this context that, even though very similar changes in postprandial gut hormone release and in bile acid levels occur after vertical sleeve gastrectomy, no effect of this procedure on EE has been found,[106,296,297] which suggests that other mechanisms may be responsible for the findings in RYGB rats and human patients.

One such potential mechanism is the gut hypertrophy that we and others observed after RYGB in rats.[124,134,250] Even though we could show that there was no difference in body composition when analyzing body fat content and total lean mass of RYGB rats and rats that were body weight-matched by food restriction, the increased mass of the gastrointestinal tract could contribute to higher EE. Saeidi et al.[250] have recently shown that the hypertrophy of the alimentary limb is associated with increased cellular proliferation, a process with very high metabolic requirements. We speculate that these adaptations to the rearrangement of the gastrointestinal tract in the RYGB procedure do not only lead to an increase in resting EE, but also explain the reported increase in DIT,[124,272,298,299] since differences in energetic requirements of the gut are more pronounced after ingestion of a meal. Importantly, gut hypertrophy seems to be specific to the RYGB surgery and has

not been reported after other bariatric procedures including sleeve gastrectomy, which supports an involvement in EE changes that have also only been observed after RYGB.

Another possibility are the changes in gut microbiota that we and others reported after RYGB.[255-258,287] These changes could lead to an increase in short-chain fatty acids, including butyrate, which are formed by microbial fermentation of complex carbohydrates. Butyrate has been shown to chronically increase EE when administered to mice by dietary supplementation[300]. Like gut hypertrophy, alterations in the bacterial flora of the gastrointestinal tract have so far only been reported in response to the RYGB procedure. Furthermore, such alterations could also explain increased DIT, since butyrate production can be expected to be highest directly after carbohydrate ingestion.

In order to further investigate the underlying causes of altered EE in RYGB rats, it could be of interest to measure EE in female ovariectomized rats with and without estrogen replacement. If increased gut hormone levels were involved in EE changes, we could potentially expect an interaction with estrogen replacement similar to the one observed for food intake (see 0). Estrogen replacement seems to increase the satiating potential of gut hormones and may have the same effect on their effects on EE. However, if the changes in EE were due to gut hypertrophy, we would not expect an interaction with estrogen replacement, unless there were a significant additional increase in the mass due to the estrogen treatment. Not enough is known about the mechanisms by which butyrate increases EE to speculate if there could be an interaction with estrogen, but we would not expect the bacterial flora and therefore butyrate production itself to be altered by estrogen.

In conclusion, the RYGB model provides a very interesting opportunity to further investigate the homeostatic systems controlling food intake, energy expenditure and bone metabolism. It could also serve to expand the emerging concept of interactions between bone remodeling, energy metabolism and adipogenesis.[197,301-307] However, the simultaneous alteration of different interacting homeostatic systems makes it very challenging to determine the potential effects or influences of one single factor.

10. Abbreviations

αMSH	α-melanocyte stimulating hormone
AEE	activity related energy expenditure
AgRP/NPY	agouti-related protein / neuropeptide Y
AL	sham operated, ad libitum fed
ARC	arcuate nucleus of the hypothalamus
BAT	brown adipose tissue
BMD	bone mineral density
BMI	body mass index
BMR	basal metabolic rate
BWM	sham operated, body weight-matched
CCK	cholecystokinin
CNS	central nervous system
DIT	diet-induced thermogenesis
DPP IV	dipeptidyl-peptidase 4
EE	energy expenditure
GLP-1	glucagon-like peptide-1
IL-6	interleukin-6
LCT	lower critical temperature
NTS	nucleus of the solitary tract
OXM	oxyntomodulin
POMC	pro-opiomelanocortin
PPARγ	peroxisome proliferator-activated gamma
PTH	parathyroid hormone
PYY	peptide YY
RQ	respiratory quotient
RYGB	Roux-en-Y gastric bypass
SNS	sympathetic nervous system
T_C	body core temperature
TEE	total daily energy expenditure
TNF-α	tumor necrosis factor-alpha
TNZ	thermoneutral zone
UCT	upper critical temperature
UCP-1	uncoupling protein-1
VCO2	carbon dioxide production
VO2	oxygen consumption
WAT	white adipose tissue

11. References

1 Gibbs, J., Young, R. C. & Smith, G. P. Cholecystokinin decreases food intake in rats. *J Comp Physiol Psychol* **84**, 488-495 (1973).

2 Muurahainen, N., Kissileff, H. R., Derogatis, A. J. & Pi-Sunyer, F. X. Effects of cholecystokinin-octapeptide (CCK-8) on food intake and gastric emptying in man. *Physiol Behav* **44**, 645-649 (1988).

3 Beglinger, C., Degen, L., Matzinger, D., D'Amato, M. & Drewe, J. Loxiglumide, a CCK-A receptor antagonist, stimulates calorie intake and hunger feelings in humans. *Am J Physiol Regul Integr Comp Physiol* **280**, R1149-1154 (2001).

4 Lutz, T. A. Pancreatic amylin as a centrally acting satiating hormone. *Curr Drug Targets* **6**, 181-189 (2005).

5 Reidelberger, R. D. *et al.* Amylin receptor blockade stimulates food intake in rats. *Am J Physiol Regul Integr Comp Physiol* **287**, R568-574, doi:10.1152/ajpregu.00213.2004 (2004).

6 Arnelo, U., Reidelberger, R., Adrian, T. E., Larsson, J. & Permert, J. Sufficiency of postprandial plasma levels of islet amyloid polypeptide for suppression of feeding in rats. *Am J Physiol* **275**, R1537-1542 (1998).

7 Williams, D. L., Baskin, D. G. & Schwartz, M. W. Evidence that intestinal glucagon-like peptide-1 plays a physiological role in satiety. *Endocrinology* **150**, 1680-1687, doi:10.1210/en.2008-1045 (2009).

8 De Silva, A. *et al.* The gut hormones PYY(3-36) and GLP-1(7-36 amide) reduce food intake and modulate brain activity in appetite centers in humans. *Cell Metab* (2011).

9 Parker, J. A. *et al.* Glucagon and GLP-1 inhibit food intake and increase c-fos expression in similar appetite regulating centres in the brainstem and amygdala. *Int J Obes (Lond)* **37**, 1391-1398, doi:10.1038/ijo.2012.227 (2013).

10 Koda, S. *et al.* The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats. *Endocrinology* **146**, 2369-2375, doi:10.1210/en.2004-1266 (2005).

11 Adams, S. H. *et al.* PYY[3-36] administration decreases the respiratory quotient and reduces adiposity in diet-induced obese mice. *J Nutr* **136**, 195-201 (2006).

12 Reidelberger, R., Haver, A. & Chelikani, P. K. Role of peptide YY(3-36) in the satiety produced by gastric delivery of macronutrients in rats. *Am J Physiol Endocrinol Metab* **304**, E944-950, doi:10.1152/ajpendo.00075.2013 (2013).

13 Vilsboll, T. *et al.* Incretin secretion in relation to meal size and body weight in healthy subjects and people with type 1 and type 2 diabetes mellitus. *J Clin Endocrinol Metab* **88**, 2706-2713 (2003).

14 Degen, L. *et al.* Effect of peptide YY3-36 on food intake in humans. *Gastroenterology* **129**, 1430-1436, doi:10.1053/j.gastro.2005.09.001 (2005).

15 Dua, A. *et al.* Leptin: a significant indicator of total body fat but not of visceral fat and insulin insensitivity in African-American women. *Diabetes* **45**, 1635-1637 (1996).

16 Havel, P. J. Mechanisms regulating leptin production: implications for control of energy balance. *Am J Clin Nutr* **70**, 305-306 (1999).

17 Maffei, M. *et al.* Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* **1**, 1155-1161 (1995).

18 Bjorntorp, P. Body fat distribution, insulin resistance, and metabolic diseases. *Nutrition* **13**, 795-803 (1997).

19 Polonsky, K. S., Given, B. D. & Van Cauter, E. Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. *J Clin Invest* **81**, 442-448, doi:10.1172/JCI113339 (1988).

20 Kwon, H. & Pessin, J. E. Adipokines mediate inflammation and insulin resistance. *Front Endocrinol (Lausanne)* **4**, 71, doi:10.3389/fendo.2013.00071 (2013).

21 Lutz, T. A. *et al.* Lesion of the area postrema/nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats. *Peptides* **19**, 309-317 (1998).

22 Johnson, A. K. & Gross, P. M. Sensory circumventricular organs and brain homeostatic pathways. *Faseb J* **7**, 678-686 (1993).

23 Moran, T. H., Baldessarini, A. R., Salorio, C. F., Lowery, T. & Schwartz, G. J. Vagal afferent and efferent contributions to the inhibition of food intake by cholecystokinin. *Am J Physiol* **272**, R1245-1251 (1997).

24 Abbott, C. R. *et al.* The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. *Brain Res* **1044**, 127-131 (2005).

25 Vine, W. *et al.* Nephrectomy decreases amylin and pramlintide clearance in rats. *Horm Metab Res* **30**, 514-517, doi:10.1055/s-2007-978923 (1998).
26 Koulischer, D., Moroder, L. & Deschodt-Lanckman, M. Degradation of cholecystokinin octapeptide, related fragments and analogs by human and rat plasma in vitro. *Regul Pept* **4**, 127-139 (1982).
27 Kieffer, T. J., McIntosh, C. H. & Pederson, R. A. Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology* **136**, 3585-3596 (1995).
28 Vilsboll, T., Agerso, H., Krarup, T. & Holst, J. J. Similar elimination rates of glucagon-like peptide-1 in obese type 2 diabetic patients and healthy subjects. *J Clin Endocrinol Metab* **88**, 220-224 (2003).
29 Berthoud, H. R., Sutton, G. M., Townsend, R. L., Patterson, L. M. & Zheng, H. Brainstem mechanisms integrating gut-derived satiety signals and descending forebrain information in the control of meal size. *Physiol Behav* **89**, 517-524, doi:10.1016/j.physbeh.2006.08.018 (2006).
30 Rinaman, L. Ascending projections from the caudal visceral nucleus of the solitary tract to brain regions involved in food intake and energy expenditure. *Brain Res* **1350**, 18-34, doi:10.1016/j.brainres.2010.03.059 (2010).
31 Varela, L. & Horvath, T. L. Leptin and insulin pathways in POMC and AgRP neurons that modulate energy balance and glucose homeostasis. *EMBO Rep* **13**, 1079-1086, doi:10.1038/embor.2012.174 (2012).
32 Meek, T. H. *et al.* Leptin action in the ventromedial hypothalamic nucleus is sufficient, but not necessary, to normalize diabetic hyperglycemia. *Endocrinology* **154**, 3067-3076, doi:10.1210/en.2013-1328 (2013).
33 Enriori, P. J., Sinnayah, P., Simonds, S. E., Garcia Rudaz, C. & Cowley, M. A. Leptin action in the dorsomedial hypothalamus increases sympathetic tone to brown adipose tissue in spite of systemic leptin resistance. *J Neurosci* **31**, 12189-12197, doi:10.1523/JNEUROSCI.2336-11.2011 (2011).
34 Dhillon, H. *et al.* Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. *Neuron* **49**, 191-203, doi:10.1016/j.neuron.2005.12.021 (2006).
35 Leinninger, G. M. Lateral thinking about leptin: a review of leptin action via the lateral hypothalamus. *Physiol Behav* **104**, 572-581, doi:10.1016/j.physbeh.2011.04.060 (2011).
36 Moran, T. H. & Ladenheim, E. E. Adiposity signaling and meal size control. *Physiol Behav* **103**, 21-24, doi:10.1016/j.physbeh.2010.11.013 (2011).
37 Hall, K. D. *et al.* Energy balance and its components: implications for body weight regulation. *Am J Clin Nutr* **95**, 989-994, doi:10.3945/ajcn.112.036350 (2012).
38 Johnstone, A. M., Murison, S. D., Duncan, J. S., Rance, K. A. & Speakman, J. R. Factors influencing variation in basal metabolic rate include fat-free mass, fat mass, age, and circulating thyroxine but not sex, circulating leptin, or triiodothyronine. *Am J Clin Nutr* **82**, 941-948 (2005).
39 Wang, Z. *et al.* Specific metabolic rates of major organs and tissues across adulthood: evaluation by mechanistic model of resting energy expenditure. *Am J Clin Nutr* **92**, 1369-1377, doi:10.3945/ajcn.2010.29885 (2010).
40 Elia, M. Organ and tissue contribution to metabolic rate. In: Energy metabolism: Tissue determinants and cellular corollaries, edited by Kinney, J. M. Tucker, H. N., Raven Press: New York. 61-77 (1992).
41 Even, P. C. & Nadkarni, N. A. Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. *Am J Physiol Regul Integr Comp Physiol* **303**, R459-476, doi:10.1152/ajpregu.00137.2012 (2012).
42 Levine, J. A. Non-exercise activity thermogenesis (NEAT). *Best Pract Res Clin Endocrinol Metab* **16**, 679-702 (2002).
43 Gordon, C. J. Temperature Regulation in Laboratory Rodents. *Cambridge University Press, New York.* (1993).
44 van Klinken, J. B., van den Berg, S. A. & van Dijk, K. W. Practical aspects of estimating energy components in rodents. *Front Physiol* **4**, 94, doi:10.3389/fphys.2013.00094 (2013).
45 Muralidhara, D. V. & Shetty, P. S. Nutritional status and spontaneous locomotor activity in the rat. *Indian J Physiol Pharmacol* **34**, 120-124 (1990).
46 Boss, O., Muzzin, P. & Giacobino, J. P. The uncoupling proteins, a review. *Eur J Endocrinol* **139**, 1-9 (1998).
47 Boss, O. *et al.* Uncoupling protein-3 expression in rodent skeletal muscle is modulated by food intake but not by changes in environmental temperature. *J Biol Chem* **273**, 5-8 (1998).
48 Tai, M. M., Castillo, P. & Pi-Sunyer, F. X. Meal size and frequency: effect on the thermic effect of food. *Am J Clin Nutr* **54**, 783-787 (1991).
49 Farshchi, H. R., Taylor, M. A. & Macdonald, I. A. Decreased thermic effect of food after an irregular compared with a regular meal pattern in healthy lean women. *Int J Obes Relat Metab Disord* **28**, 653-660, doi:10.1038/sj.ijo.0802616 (2004).

50 Denzer, C. M. & Young, J. C. The effect of resistance exercise on the thermic effect of food. *Int J Sport Nutr Exerc Metab* **13**, 396-402 (2003).
51 Stob, N. R., Bell, C., van Baak, M. A. & Seals, D. R. Thermic effect of food and beta-adrenergic thermogenic responsiveness in habitually exercising and sedentary healthy adult humans. *J Appl Physiol* **103**, 616-622, doi:10.1152/japplphysiol.01434.2006 (2007).
52 de Jonge, L. & Bray, G. A. The thermic effect of food and obesity: a critical review. *Obes Res* **5**, 622-631 (1997).
53 Watanabe, T. *et al.* Relationships between thermic effect of food, insulin resistance and autonomic nervous activity. *J Med Invest* **53**, 153-158 (2006).
54 Tappy, L. Thermic effect of food and sympathetic nervous system activity in humans. *Reprod Nutr Dev* **36**, 391-397 (1996).
55 Cassaglia, P. A., Hermes, S. M., Aicher, S. A. & Brooks, V. L. Insulin acts in the arcuate nucleus to increase lumbar sympathetic nerve activity and baroreflex function in rats. *J Physiol* **589**, 1643-1662, doi:10.1113/jphysiol.2011.205575 (2011).
56 Elefteriou, F. *et al.* Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature* **434**, 514-520 (2005).
57 Osaka, T., Endo, M., Yamakawa, M. & Inoue, S. Energy expenditure by intravenous administration of glucagon-like peptide-1 mediated by the lower brainstem and sympathoadrenal system. *Peptides* **26**, 1623-1631, doi:10.1016/j.peptides.2005.02.016 (2005).
58 Osaka, T., Tsukamoto, A., Koyama, Y. & Inoue, S. Central and peripheral administration of amylin induces energy expenditure in anesthetized rats. *Peptides* **29**, 1028-1035, doi:10.1016/j.peptides.2008.02.002 (2008).
59 Kaiyala, K. J. & Ramsay, D. S. Direct animal calorimetry, the underused gold standard for quantifying the fire of life. *Comp Biochem Physiol A Mol Integr Physiol* **158**, 252-264, doi:10.1016/j.cbpa.2010.04.013 (2011).
60 Saltzman, E. & Roberts, S. B. The role of energy expenditure in energy regulation: findings from a decade of research. *Nutr Rev* **53**, 209-220 (1995).
61 Kaiyala, K. J. *et al.* Identification of body fat mass as a major determinant of metabolic rate in mice. *Diabetes* **59**, 1657-1666 (2010).
62 Kaiyala, K. J. & Schwartz, M. W. Toward a more complete (and less controversial) understanding of energy expenditure and its role in obesity pathogenesis. *Diabetes* **60**, 17-23, doi:10.2337/db10-0909 (2011).
63 Tschop, M. H. *et al.* A guide to analysis of mouse energy metabolism. *Nat Methods* **9**, 57-63, doi:10.1038/nmeth.1806 (2012).
64 Butler, A. A. & Kozak, L. P. A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes. *Diabetes* **59**, 323-329 (2010).
65 Enerback, S. *et al.* Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* **387**, 90-94, doi:10.1038/387090a0 (1997).
66 Liu, X. *et al.* Paradoxical resistance to diet-induced obesity in UCP1-deficient mice. *J Clin Invest* **111**, 399-407, doi:10.1172/JCI15737 (2003).
67 Kontani, Y. *et al.* UCP1 deficiency increases susceptibility to diet-induced obesity with age. *Aging Cell* **4**, 147-155, doi:10.1111/j.1474-9726.2005.00157.x (2005).
68 Feldmann, H. M., Golozoubova, V., Cannon, B. & Nedergaard, J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab* **9**, 203-209, doi:10.1016/j.cmet.2008.12.014 (2009).
69 Cannon, B. & Nedergaard, J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J Exp Biol* **214**, 242-253, doi:10.1242/jeb.050989 (2011).
70 Karp, C. L. Unstressing intemperate models: how cold stress undermines mouse modeling. *J Exp Med* **209**, 1069-1074, doi:10.1084/jem.20120988 (2012).
71 Overton, J. M. Phenotyping small animals as models for the human metabolic syndrome: thermoneutrality matters. *Int J Obes (Lond)* **34 Suppl 2**, S53-58, doi:10.1038/ijo.2010.240 (2010).
72 Herrington, L. P. The heat regulation of small laboratory animals at various environmental temperatures. *Am J Physiol* **129**, 123-139 (1940).
73 Gordon, C. J. Relationship between autonomic and behavioral thermoregulation in the mouse. *Physiol Behav* **34**, 687-690 (1985).
74 Clarkson, D. P., Schatte, C. L. & Jordan, J. P. Thermal neutral temperature of rats in helium-oxygen, argon-oxygen, and air. *Am J Physiol* **222**, 1494-1498 (1972).
75 Gordon, C. J. Relationship between preferred ambient temperature and autonomic thermoregulatory function in rat. *Am J Physiol* **252**, R1130-1137 (1987).

76 Poole, S. & Stephenson, J. D. Body temperature regulation and thermoneutrality in rats. *Q J Exp Physiol Cogn Med Sci* **62**, 143-149 (1977).
77 Woods, S. C. & Langhans, W. Inconsistencies in the assessment of food intake. *Am J Physiol Endocrinol Metab* **303**, E1408-1418, doi:10.1152/ajpendo.00415.2012 (2012).
78 Sanghez, V. *et al.* Psychosocial stress induces hyperphagia and exacerbates diet-induced insulin resistance and the manifestations of the Metabolic Syndrome. *Psychoneuroendocrinology*, doi:10.1016/j.psyneuen.2013.07.022 (2013).
79 Siervo, M., Wells, J. C. & Cizza, G. The contribution of psychosocial stress to the obesity epidemic: an evolutionary approach. *Horm Metab Res* **41**, 261-270, doi:10.1055/s-0028-1119377 (2009).
80 von Deneen, K. M. & Liu, Y. Obesity as an addiction: Why do the obese eat more? *Maturitas* **68**, 342-345, doi:10.1016/j.maturitas.2011.01.018 (2011).
81 Berthoud, H. R., Lenard, N. R. & Shin, A. C. Food reward, hyperphagia, and obesity. *Am J Physiol Regul Integr Comp Physiol* **300**, R1266-1277, doi:10.1152/ajpregu.00028.2011 (2011).
82 Taghva, A., Corrigan, J. D. & Rezai, A. R. Obesity and brain addiction circuitry: implications for deep brain stimulation. *Neurosurgery* **71**, 224-238, doi:10.1227/NEU.0b013e31825972ab (2012).
83 Benyshek, D. C. The developmental origins of obesity and related health disorders--prenatal and perinatal factors. *Coll Antropol* **31**, 11-17 (2007).
84 McMillen, I. C., Muhlhausler, B. S., Duffield, J. A. & Yuen, B. S. Prenatal programming of postnatal obesity: fetal nutrition and the regulation of leptin synthesis and secretion before birth. *Proc Nutr Soc* **63**, 405-412 (2004).
85 Gonzalez-Bulnes, A. & Ovilo, C. Genetic basis, nutritional challenges and adaptive responses in the prenatal origin of obesity and type-2 diabetes. *Curr Diabetes Rev* **8**, 144-154 (2012).
86 http://www.who.int.
87 http://www.who.int/mediacentre/factsheets/fs311/en/index.html.
88 Wadden, T. A., McGuckin, B. G., Rothman, R. A. & Sargent, S. L. Lifestyle modification in the management of obesity. *J Gastrointest Surg* **7**, 452-463 (2003).
89 Perri, M. G. & Corsica, J. A. Improving the maintenance of weight loss in behavioral treatment of obesity. In: Wadden, T. A. & Stunkard, A. J. (Eds.) Handbook of obesity treatment. New York: Guilford Press. . 357-379 (2002).
90 Wadden, T. A., Foster, G. D., Letizia, K. A. & Mullen, J. L. Long-term effects of dieting on resting metabolic rate in obese outpatients. *Jama* **264**, 707-711 (1990).
91 Rucker, D., Padwal, R., Li, S. K., Curioni, C. & Lau, D. C. Long term pharmacotherapy for obesity and overweight: updated meta-analysis. *Bmj* **335**, 1194-1199, doi:10.1136/bmj.39385.413113.25 (2007).
92 Padwal, R., Li, S. K. & Lau, D. C. Long-term pharmacotherapy for obesity and overweight. *Cochrane Database Syst Rev*, CD004094, doi:10.1002/14651858.CD004094.pub2 (2004).
93 James, W. P. *et al.* Effect of sibutramine on cardiovascular outcomes in overweight and obese subjects. *N Engl J Med* **363**, 905-917, doi:10.1056/NEJMoa1003114 (2010).
94 http:// www.ema.europa.eu/humandocs/PDFs/ EPAR /zimulti/3956009en.pdf Last accessed: October 14.
95 Misra, M. Obesity pharmacotherapy: current perspectives and future directions. *Curr Cardiol Rev* **9**, 33-54 (2013).
96 Holes-Lewis, K. A., Malcolm, R. & O'Neil, P. M. Pharmacotherapy of obesity: clinical treatments and considerations. *Am J Med Sci* **345**, 284-288, doi:10.1097/MAJ.0b013e31828abcfd (2013).
97 Xanthakos, S. A. & Inge, T. H. Nutritional consequences of bariatric surgery. *Curr Opin Clin Nutr Metab Care* **9**, 489-496, doi:10.1097/01.mco.0000232913.07355.cf (2006).
98 Dixon, J. B., Straznicky, N. E., Lambert, E. A., Schlaich, M. P. & Lambert, G. W. Surgical approaches to the treatment of obesity. *Nat Rev Gastroenterol Hepatol* **8**, 429-437, doi:10.1038/nrgastro.2011.112 (2011).
99 Cummings, D. E., Overduin, J. & Foster-Schubert, K. E. Gastric bypass for obesity: mechanisms of weight loss and diabetes resolution. *J Clin Endocrinol Metab* **89**, 2608-2615, doi:10.1210/jc.2004-0433 (2004).
100 Yin, D. P. *et al.* Assessment of different bariatric surgeries in the treatment of obesity and insulin resistance in mice. *Ann Surg* **254**, 73-82 (2011).
101 Oldfield, B. J., Stefanidis, A. & Kampe, J. Mechanisms underlying the effectiveness of the adjustable gastric band. *19th Annual Meeting of the Society for the Study of Ingestive Behavior, Clearwater, Florida* (2011).
102 Ahmed, A. R. *et al.* Laparoscopic Roux-en-Y gastric bypass and its early effect on blood pressure. *Obes Surg* **19**, 845-849, doi:10.1007/s11695-008-9671-z (2009).
103 Cohen, R. V. *et al.* Effects of gastric bypass surgery in patients with type 2 diabetes and only mild obesity. *Diabetes Care* **35**, 1420-1428, doi:10.2337/dc11-2289 (2012).

104 Buchwald, H. & Oien, D. M. Metabolic/bariatric surgery Worldwide 2008. *Obes Surg* **19**, 1605-1611 (2009).
105 Himpens, J., Dapri, G. & Cadiere, G. B. A prospective randomized study between laparoscopic gastric banding and laparoscopic isolated sleeve gastrectomy: results after 1 and 3 years. *Obes Surg* **16**, 1450-1456, doi:10.1381/096089206778869933 (2006).
106 Cummings, B. P. *et al.* Vertical sleeve gastrectomy improves glucose and lipid metabolism and delays diabetes onset in UCD-T2DM rats. *Endocrinology* **153**, 3620-3632, doi:10.1210/en.2012-1131 (2012).
107 Nannipieri, M. *et al.* Roux-en-Y Gastric Bypass and Sleeve Gastrectomy: Mechanisms of Diabetes Remission and Role of Gut Hormones. *J Clin Endocrinol Metab*, doi:10.1210/jc.2013-2538 (2013).
108 Catheline, J. M. *et al.* Five-year results of sleeve gastrectomy. *J Visc Surg*, doi:10.1016/j.jviscsurg.2013.08.008 (2013).
109 Shah, M., Simha, V. & Garg, A. Review: long-term impact of bariatric surgery on body weight, comorbidities, and nutritional status. *J Clin Endocrinol Metab* **91**, 4223-4231, doi:10.1210/jc.2006-0557 (2006).
110 Brolin, R. E., LaMarca, L. B., Kenler, H. A. & Cody, R. P. Malabsorptive gastric bypass in patients with superobesity. *J Gastrointest Surg* **6**, 195-203 (2002).
111 MacLean, L. D., Rhode, B. M. & Nohr, C. W. Long- or short-limb gastric bypass? *J Gastrointest Surg* **5**, 525-530 (2001).
112 Miras, A. D. & le Roux, C. W. Mechanisms underlying weight loss after bariatric surgery. *Nat Rev Gastroenterol Hepatol* **10**, 575-584, doi:10.1038/nrgastro.2013.119 (2013).
113 Le Roux, C. W. *et al.* Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. *Ann Surg* **243**, 108-114 (2006).
114 Ashrafian, H. & le Roux, C. W. Metabolic surgery and gut hormones - a review of bariatric entero-humoral modulation. *Physiol Behav* **97**, 620-631, doi:10.1016/j.physbeh.2009.03.012 (2009).
115 Shin, A. C., Zheng, H., Townsend, R. L., Sigalet, D. L. & Berthoud, H. R. Meal-induced hormone responses in a rat model of Roux-en-Y gastric bypass surgery. *Endocrinology* **151**, 1588-1597, doi:10.1210/en.2009-1332 (2010).
116 Bueter, M. *et al.* Vagal sparing surgical technique but not stoma size affects body weight loss in rodent model of gastric bypass. *Obes Surg* **20**, 616-622, doi:10.1007/s11695-010-0075-5 (2010).
117 Le Roux, C. W. *et al.* Gastric bypass reduces fat intake and preference. *Am J Physiol Regul Integr Comp Physiol* **301**, R1057-1066, doi:10.1152/ajpregu.00139.2011 (2011).
118 Zheng, H. *et al.* Meal patterns, satiety, and food choice in a rat model of Roux-en-Y gastric bypass surgery. *Am J Physiol Regul Integr Comp Physiol* **297**, R1273-1282, doi:10.1152/ajpregu.00343.2009 (2009).
119 Bueter, M. *et al.* Alterations of sucrose preference after Roux-en-Y gastric bypass. *Physiol Behav* **104**, 709-721, doi:10.1016/j.physbeh.2011.07.025 (2011).
120 Hajnal, A. *et al.* Gastric bypass surgery alters behavioral and neural taste functions for sweet taste in obese rats. *Am J Physiol Gastrointest Liver Physiol* **299**, G967-979, doi:10.1152/ajpgi.00070.2010 (2010).
121 Tichansky, D. S. *et al.* Decrease in sweet taste in rats after gastric bypass surgery. *Surg Endosc* **25**, 1176-1181, doi:10.1007/s00464-010-1335-0 (2011).
122 Miras, A. D. *et al.* Gastric bypass surgery for obesity decreases the reward value of a sweet-fat stimulus as assessed in a progressive ratio task. *Am J Clin Nutr* **96**, 467-473, doi:10.3945/ajcn.112.036921 (2012).
123 Aron-Wisnewsky, J., Dore, J. & Clement, K. The importance of the gut microbiota after bariatric surgery. *Nat Rev Gastroenterol Hepatol* **9**, 590-598, doi:10.1038/nrgastro.2012.161 (2012).
124 Bueter, M. *et al.* Gastric bypass increases energy expenditure in rats. *Gastroenterology* **138**, 1845-1853, doi:10.1053/j.gastro.2009.11.012 (2010).
125 Stylopoulos, N., Hoppin, A. G. & Kaplan, L. M. Roux-en-Y gastric bypass enhances energy expenditure and extends lifespan in diet-induced obese rats. *Obesity (Silver Spring)* **17**, 1839-1847, doi:10.1038/oby.2009.207 (2009).
126 Kinzig, K. P., D'Alessio, D. A. & Seeley, R. J. The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. *J Neurosci* **22**, 10470-10476 (2002).
127 Chelikani, P. K., Haver, A. C. & Reidelberger, R. D. Intravenous infusion of glucagon-like peptide-1 potently inhibits food intake, sham feeding, and gastric emptying in rats. *Am J Physiol Regul Integr Comp Physiol* **288**, R1695-1706, doi:10.1152/ajpregu.00870.2004 (2005).
128 Gibbons, C. *et al.* Comparison of postprandial profiles of ghrelin, active GLP-1, and total PYY to meals varying in fat and carbohydrate and their association with hunger and the phases of satiety. *J Clin Endocrinol Metab* **98**, E847-855, doi:10.1210/jc.2012-3835 (2013).

129 Flint, A., Raben, A., Ersboll, A. K., Holst, J. J. & Astrup, A. The effect of physiological levels of glucagon-like peptide-1 on appetite, gastric emptying, energy and substrate metabolism in obesity. *Int J Obes Relat Metab Disord* **25**, 781-792 (2001).
130 Flint, A., Raben, A., Rehfeld, J. F., Holst, J. J. & Astrup, A. The effect of glucagon-like peptide-1 on energy expenditure and substrate metabolism in humans. *Int J Obes Relat Metab Disord* **24**, 288-298 (2000).
131 Conarello, S. L. *et al.* Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin resistance. *Proc Natl Acad Sci U S A* **100**, 6825-6830, doi:10.1073/pnas.0631828100 (2003).
132 Falken, Y., Hellstrom, P. M., Holst, J. J. & Naslund, E. Changes in glucose homeostasis after Roux-en-Y gastric bypass surgery for obesity at day three, two months, and one year after surgery: role of gut peptides. *J Clin Endocrinol Metab* **96**, 2227-2235 (2011).
133 Laferrere, B. *et al.* Rise of oxyntomodulin in response to oral glucose after gastric bypass surgery in patients with type 2 diabetes. *J Clin Endocrinol Metab* **95**, 4072-4076, doi:10.1210/jc.2009-2767 (2010).
134 Le Roux, C. W. *et al.* Gut hypertrophy after gastric bypass is associated with increased glucagon-like peptide 2 and intestinal crypt cell proliferation. *Ann Surg* **252**, 50-56, doi:10.1097/SLA.0b013e3181d3d21f (2010).
135 Dakin, C. L. *et al.* Repeated ICV administration of oxyntomodulin causes a greater reduction in body weight gain than in pair-fed rats. *Am J Physiol Endocrinol Metab* **283**, E1173-1177, doi:10.1152/ajpendo.00233.2002 (2002).
136 Wynne, K. *et al.* Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial. *Int J Obes (Lond)* **30**, 1729-1736, doi:10.1038/sj.ijo.0803344 (2006).
137 Drucker, D. J. Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis. *Mol Endocrinol* **17**, 161-171 (2003).
138 Tsai, C. H., Hill, M., Asa, S. L., Brubaker, P. L. & Drucker, D. J. Intestinal growth-promoting properties of glucagon-like peptide-2 in mice. *Am J Physiol* **273**, E77-84 (1997).
139 Tsai, C. H., Hill, M. & Drucker, D. J. Biological determinants of intestinotrophic properties of GLP-2 in vivo. *Am J Physiol* **272**, G662-668 (1997).
140 Cant, J. P., McBride, B. W. & Croom, W. J., Jr. The regulation of intestinal metabolism and its impact on whole animal energetics. *J Anim Sci* **74**, 2541-2553 (1996).
141 Korner, J. *et al.* Effects of Roux-en-Y gastric bypass surgery on fasting and postprandial concentrations of plasma ghrelin, peptide YY, and insulin. *J Clin Endocrinol Metab* **90**, 359-365 (2005).
142 Morinigo, R. *et al.* Glucagon-like peptide-1, peptide YY, hunger, and satiety after gastric bypass surgery in morbidly obese subjects. *J Clin Endocrinol Metab* **91**, 1735-1740, doi:10.1210/jc.2005-0904 (2006).
143 Borg, C. M. *et al.* Progressive rise in gut hormone levels after Roux-en-Y gastric bypass suggests gut adaptation and explains altered satiety. *Br J Surg* **93**, 210-215 (2006).
144 Le Roux, C. W. *et al.* Gut hormones as mediators of appetite and weight loss after Roux-en-Y gastric bypass. *Ann Surg* **246**, 780-785, doi:10.1097/SLA.0b013e3180caa3e3 (2007).
145 Rodieux, F., Giusti, V., D'Alessio, D. A., Suter, M. & Tappy, L. Effects of gastric bypass and gastric banding on glucose kinetics and gut hormone release. *Obesity (Silver Spring)* **16**, 298-305, doi:10.1038/oby.2007.83 (2008).
146 Vidal, J. *et al.* Long-term effects of Roux-en-Y gastric bypass surgery on plasma glucagon-like peptide-1 and islet function in morbidly obese subjects. *J Clin Endocrinol Metab* **94**, 884-891, doi:10.1210/jc.2008-1620 (2009).
147 Clements, R. H., Gonzalez, Q. H., Long, C. I., Wittert, G. & Laws, H. L. Hormonal changes after Roux-en Y gastric bypass for morbid obesity and the control of type-II diabetes mellitus. *Am Surg* **70**, 1-4; discussion 4-5 (2004).
148 Whitson, B. A. *et al.* Entero-endocrine changes after gastric bypass in diabetic and nondiabetic patients: a preliminary study. *J Surg Res* **141**, 31-39, doi:10.1016/j.jss.2007.02.022 (2007).
149 Reinehr, T. *et al.* Peptide YY and glucagon-like peptide-1 in morbidly obese patients before and after surgically induced weight loss. *Obes Surg* **17**, 1571-1577, doi:10.1007/s11695-007-9323-8 (2007).
150 Rothwell, N. J. & Stock, M. J. A role for brown adipose tissue in diet-induced thermogenesis. *Nature* **281**, 31-35 (1979).
151 Cannon, B. & Nedergaard, J. Brown adipose tissue: function and physiological significance. *Physiol Rev* **84**, 277-359, doi:10.1152/physrev.00015.2003 (2004).
152 Nicholls, D. G. & Locke, R. M. Thermogenic mechanisms in brown fat. *Physiol Rev* **64**, 1-64 (1984).

153 Ricquier, D. Respiration uncoupling and metabolism in the control of energy expenditure. *Proc Nutr Soc* **64**, 47-52 (2005).
154 Lee, P., Greenfield, J. R., Ho, K. K. & Fulham, M. J. A critical appraisal of the prevalence and metabolic significance of brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* **299**, E601-606, doi:10.1152/ajpendo.00298.2010 (2010).
155 Yilmaz, Y. *et al.* Association between the presence of brown adipose tissue and non-alcoholic fatty liver disease in adult humans. *Aliment Pharmacol Ther* **34**, 318-323 (2011).
156 Nedergaard, J., Bengtsson, T. & Cannon, B. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* **293**, E444-452, doi:10.1152/ajpendo.00691.2006 (2007).
157 Cypess, A. M. *et al.* Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* **360**, 1509-1517, doi:10.1056/NEJMoa0810780 (2009).
158 Saito, M. *et al.* High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* **58**, 1526-1531, doi:10.2337/db09-0530 (2009).
159 Zingaretti, M. C. *et al.* The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. *Faseb J* **23**, 3113-3120, doi:10.1096/fj.09-133546 (2009).
160 Kajimura, S., Seale, P. & Spiegelman, B. M. Transcriptional control of brown fat development. *Cell Metab* **11**, 257-262, doi:10.1016/j.cmet.2010.03.005 (2010).
161 Nedergaard, J. & Cannon, B. The changed metabolic world with human brown adipose tissue: therapeutic visions. *Cell Metab* **11**, 268-272, doi:10.1016/j.cmet.2010.03.007 (2010).
162 Timmons, J. A. *et al.* Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc Natl Acad Sci U S A* **104**, 4401-4406, doi:10.1073/pnas.0610615104 (2007).
163 Seale, P. *et al.* PRDM16 controls a brown fat/skeletal muscle switch. *Nature* **454**, 961-967, doi:10.1038/nature07182 (2008).
164 Cousin, B. *et al.* Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J Cell Sci* **103 (Pt 4)**, 931-942 (1992).
165 Collins, S., Daniel, K. W., Petro, A. E. & Surwit, R. S. Strain-specific response to beta 3-adrenergic receptor agonist treatment of diet-induced obesity in mice. *Endocrinology* **138**, 405-413 (1997).
166 Himms-Hagen, J. *et al.* Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am J Physiol Cell Physiol* **279**, C670-681 (2000).
167 Nedergaard, J., Petrovic, N., Lindgren, E. M., Jacobsson, A. & Cannon, B. PPARgamma in the control of brown adipocyte differentiation. *Biochim Biophys Acta* **1740**, 293-304, doi:10.1016/j.bbadis.2005.02.003 (2005).
168 Veyrat-Durebex, C., Poher, A. L., Caillon, A., Montet, X. & Rohner-Jeanrenaud, F. Alterations in lipid metabolism and thermogenesis with emergence of brown adipocytes in white adipose tissue in diet-induced obesity-resistant Lou/C rats. *Am J Physiol Endocrinol Metab* **300**, E1146-1157, doi:10.1152/ajpendo.00575.2010 (2011).
169 Perrin, D. *et al.* Sympathetic and brain monoaminergic regulation of energy balance in obesity-resistant rats (Lou/C). *Auton Neurosci* **109**, 1-9, doi:10.1016/j.autneu.2003.08.008 (2003).
170 Reid, I. R. Relationships among body mass, its components, and bone. *Bone* **31**, 547-555 (2002).
171 Wang, M. C. *et al.* The relative contributions of lean tissue mass and fat mass to bone density in young women. *Bone* **37**, 474-481, doi:10.1016/j.bone.2005.04.038 (2005).
172 Felson, D. T., Zhang, Y., Hannan, M. T. & Anderson, J. J. Effects of weight and body mass index on bone mineral density in men and women: the Framingham study. *J Bone Miner Res* **8**, 567-573, doi:10.1002/jbmr.5650080507 (1993).
173 Ehrlich, P. J. & Lanyon, L. E. Mechanical strain and bone cell function: a review. *Osteoporos Int* **13**, 688-700, doi:10.1007/s001980200095 (2002).
174 Weiler, H. A. *et al.* Percent body fat and bone mass in healthy Canadian females 10 to 19 years of age. *Bone* **27**, 203-207 (2000).
175 Goulding, A. *et al.* Overweight and obese children have low bone mass and area for their weight. *Int J Obes Relat Metab Disord* **24**, 627-632 (2000).
176 Blum, M. *et al.* Leptin, body composition and bone mineral density in premenopausal women. *Calcif Tissue Int* **73**, 27-32 (2003).
177 Hsu, Y. H. *et al.* Relation of body composition, fat mass, and serum lipids to osteoporotic fractures and bone mineral density in Chinese men and women. *Am J Clin Nutr* **83**, 146-154 (2006).
178 Pollock, N. K. *et al.* Is adiposity advantageous for bone strength? A peripheral quantitative computed tomography study in late adolescent females. *Am J Clin Nutr* **86**, 1530-1538 (2007).

179 Hypponen, E. & Power, C. Hypovitaminosis D in British adults at age 45 y: nationwide cohort study of dietary and lifestyle predictors. *Am J Clin Nutr* **85**, 860-868 (2007).
180 Wortsman, J., Matsuoka, L. Y., Chen, T. C., Lu, Z. & Holick, M. F. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr* **72**, 690-693 (2000).
181 Compston, J. E. *et al.* Vitamin D status and bone histomorphometry in gross obesity. *Am J Clin Nutr* **34**, 2359-2363 (1981).
182 Flancbaum, L., Belsley, S., Drake, V., Colarusso, T. & Tayler, E. Preoperative nutritional status of patients undergoing Roux-en-Y gastric bypass for morbid obesity. *J Gastrointest Surg* **10**, 1033-1037, doi:10.1016/j.gassur.2006.03.004 (2006).
183 Carlin, A. M. *et al.* Prevalence of vitamin D depletion among morbidly obese patients seeking gastric bypass surgery. *Surg Obes Relat Dis* **2**, 98-103; discussion 104, doi:10.1016/j.soard.2005.12.001 (2006).
184 Poole, K. E. & Reeve, J. Parathyroid hormone - a bone anabolic and catabolic agent. *Curr Opin Pharmacol* **5**, 612-617, doi:10.1016/j.coph.2005.07.004 (2005).
185 Hotamisligil, G. S. Inflammation and metabolic disorders. *Nature* **444**, 860-867, doi:10.1038/nature05485 (2006).
186 Kern, P. A., Ranganathan, S., Li, C., Wood, L. & Ranganathan, G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab* **280**, E745-751 (2001).
187 Hotamisligil, G. S., Shargill, N. S. & Spiegelman, B. M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**, 87-91 (1993).
188 Tzanavari, T., Giannogonas, P. & Karalis, K. P. TNF-alpha and obesity. *Curr Dir Autoimmun* **11**, 145-156 (2010).
189 Pradhan, A. D., Manson, J. E., Rifai, N., Buring, J. E. & Ridker, P. M. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *Jama* **286**, 327-334 (2001).
190 Mundy, G. R. Osteoporosis and inflammation. *Nutr Rev* **65**, S147-151 (2007).
191 Vargas, S. J. *et al.* Interleukin-6 expression and histomorphometry of bones from mice deficient in receptors for interleukin-1 or tumor necrosis factor. *J Bone Miner Res* **11**, 1736-1744, doi:10.1002/jbmr.5650111117 (1996).
192 Kimble, R. B. *et al.* Simultaneous block of interleukin-1 and tumor necrosis factor is required to completely prevent bone loss in the early postovariectomy period. *Endocrinology* **136**, 3054-3061 (1995).
193 Shimizu, H. *et al.* Serum leptin concentration is associated with total body fat mass, but not abdominal fat distribution. *Int J Obes Relat Metab Disord* **21**, 536-541 (1997).
194 Canavan, B. *et al.* Effects of physiological leptin administration on markers of inflammation, platelet activation, and platelet aggregation during caloric deprivation. *J Clin Endocrinol Metab* **90**, 5779-5785, doi:10.1210/jc.2005-0780 (2005).
195 Van Dielen, F. M. *et al.* Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. *Int J Obes Relat Metab Disord* **25**, 1759-1766, doi:10.1038/sj.ijo.0801825 (2001).
196 Yadav, V. K. *et al.* A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell* **138**, 976-989, doi:10.1016/j.cell.2009.06.051 (2009).
197 Karsenty, G. & Oury, F. The central regulation of bone mass, the first link between bone remodeling and energy metabolism. *J Clin Endocrinol Metab* **95**, 4795-4801 (2010).
198 Ducy, P. *et al.* Leptin inhibits bone formation through a hypothalamic relay: A central control of bone mass. *Cell* **100**, 197-207 (2000).
199 Oury, F. & Karsenty, G. Towards a serotonin-dependent leptin roadmap in the brain. *Trends Endocrinol Metab* **22**, 382-387 (2011).
200 Steppan, C. M., Crawford, D. T., Chidsey-Frink, K. L., Ke, H. & Swick, A. G. Leptin is a potent stimulator of bone growth in ob/ob mice. *Regul Pept* **92**, 73-78 (2000).
201 Burguera, B. *et al.* Leptin reduces ovariectomy-induced bone loss in rats. *Endocrinology* **142**, 3546-3553 (2001).
202 Cornish, J. *et al.* Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo. *J Endocrinol* **175**, 405-415 (2002).
203 Bartell, S. M. *et al.* Central (ICV) leptin injection increases bone formation, bone mineral density, muscle mass, serum IGF-1, and the expression of osteogenic genes in leptin-deficient ob/ob mice. *J Bone Miner Res* **26**, 1710-1720, doi:10.1002/jbmr.406 (2011).
204 Jackson, M. A., Iwaniec, U. T., Turner, R. T., Wronski, T. J. & Kalra, S. P. Effects of increased hypothalamic leptin gene expression on ovariectomy-induced bone loss in rats. *Peptides* **32**, 1575-1580 (2011).

205 Conroy, R. *et al.* Leptin administration does not prevent the bone mineral metabolism changes induced by weight loss. *Metabolism* **60**, 1222-1226, doi:10.1016/j.metabol.2011.02.010 (2011).
206 Barbour, K. E. *et al.* The effects of adiponectin and leptin on changes in bone mineral density. *Osteoporos Int* **23**, 1699-1710, doi:10.1007/s00198-011-1768-x (2012).
207 Zemel, M. B. Regulation of adiposity and obesity risk by dietary calcium: mechanisms and implications. *J Am Coll Nutr* **21**, 146S-151S (2002).
208 Papakonstantinou, E., Flatt, W. P., Huth, P. J. & Harris, R. B. High dietary calcium reduces body fat content, digestibility of fat, and serum vitamin D in rats. *Obes Res* **11**, 387-394, doi:10.1038/oby.2003.52 (2003).
209 Zemel, M. B., Thompson, W., Milstead, A., Morris, K. & Campbell, P. Calcium and dairy acceleration of weight and fat loss during energy restriction in obese adults. *Obes Res* **12**, 582-590 (2004).
210 Skinner, J. D., Bounds, W., Carruth, B. R. & Ziegler, P. Longitudinal calcium intake is negatively related to children's body fat indexes. *J Am Diet Assoc* **103**, 1626-1631, doi:10.1016/j.jada.2003.09.018 (2003).
211 Jacobsen, R., Lorenzen, J. K., Toubro, S., Krog-Mikkelsen, I. & Astrup, A. Effect of short-term high dietary calcium intake on 24-h energy expenditure, fat oxidation, and fecal fat excretion. *Int J Obes (Lond)* **29**, 292-301, doi:10.1038/sj.ijo.0802785 (2005).
212 Welberg, J. W. *et al.* Effects of supplemental dietary calcium on quantitative and qualitative fecal fat excretion in man. *Ann Nutr Metab* **38**, 185-191 (1994).
213 Zernicke, R. F., Salem, G. J., Barnard, R. J. & Schramm, E. Long-term, high-fat-sucrose diet alters rat femoral neck and vertebral morphology, bone mineral content, and mechanical properties. *Bone* **16**, 25-31 (1995).
214 Salem, G. J., Zernicke, R. F. & Barnard, R. J. Diet-related changes in mechanical properties of rat vertebrae. *Am J Physiol* **262**, R318-321 (1992).
215 Xiao, Y. *et al.* Effects of duodenal redox status on calcium absorption and related genes expression in high-fat diet-fed mice. *Nutrition* **26**, 1188-1194, doi:10.1016/j.nut.2009.11.021 (2010).
216 Gimble, J. M., Robinson, C. E., Wu, X. & Kelly, K. A. The function of adipocytes in the bone marrow stroma: an update. *Bone* **19**, 421-428 (1996).
217 Gimble, J. M., Zvonic, S., Floyd, Z. E., Kassem, M. & Nuttall, M. E. Playing with bone and fat. *J Cell Biochem* **98**, 251-266, doi:10.1002/jcb.20777 (2006).
218 Maurin, A. C. *et al.* Influence of mature adipocytes on osteoblast proliferation in human primary cocultures. *Bone* **26**, 485-489, doi:10.1016/S8756-3282(00)00252-0 (2000).
219 Duque, G. Bone and fat connection in aging bone. *Curr Opin Rheumatol* **20**, 429-434 (2008).
220 Chamberlain, G., Fox, J., Ashton, B. & Middleton, J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* **25**, 2739-2749, doi:10.1634/stemcells.2007-0197 (2007).
221 Zhou, S. *et al.* Age-related intrinsic changes in human bone-marrow-derived mesenchymal stem cells and their differentiation to osteoblasts. *Aging Cell* **7**, 335-343 (2008).
222 Rosen, C. J. & Bouxsein, M. L. Mechanisms of disease: is osteoporosis the obesity of bone? *Nat Clin Pract Rheumatol* **2**, 35-43, doi:10.1038/ncprheum0070 (2006).
223 Wan, Y., Chong, L. W. & Evans, R. M. PPAR-gamma regulates osteoclastogenesis in mice. *Nat Med* **13**, 1496-1503, doi:10.1038/nm1672 (2007).
224 Wei, W. *et al.* Osteoclast progenitors reside in the peroxisome proliferator-activated receptor gamma-expressing bone marrow cell population. *Mol Cell Biol* **31**, 4692-4705, doi:10.1128/MCB.05979-11 (2011).
225 Yaturu, S., Bryant, B. & Jain, S. K. Thiazolidinedione treatment decreases bone mineral density in type 2 diabetic men. *Diabetes Care* **30**, 1574-1576, doi:10.2337/dc06-2606 (2007).
226 Riche, D. M. & King, S. T. Bone loss and fracture risk associated with thiazolidinedione therapy. *Pharmacotherapy* **30**, 716-727, doi:10.1592/phco.30.7.716 (2010).
227 Joao Cabrera, E., Valezi, A. C., Delfino, V. D., Lavado, E. L. & Barbosa, D. S. Reduction in plasma levels of inflammatory and oxidative stress indicators after Roux-en-Y gastric bypass. *Obes Surg* **20**, 42-49 (2010).
228 Valezi, A. C. *et al.* Roux-en-Y gastric bypass and inflammatory activity of the adipose tissue. *Rev Col Bras Cir* **38**, 161-166 (2011).
229 Miller, G. D., Nicklas, B. J. & Fernandez, A. Serial changes in inflammatory biomarkers after Roux-en-Y gastric bypass surgery. *Surg Obes Relat Dis* **7**, 618-624, doi:10.1016/j.soard.2011.03.006 (2011).
230 Thomas, J. R. & Marcus, E. High and low fat food selection with reported frequency intolerance following Roux-en-Y gastric bypass. *Obes Surg* **18**, 282-287 (2008).
231 Nogues, X. *et al.* [Bone mass loss after sleeve gastrectomy: a prospective comparative study with gastric bypass]. *Cir Esp* **88**, 103-109, doi:10.1016/j.ciresp.2010.04.008 (2010).

232 Fleischer, J. *et al.* The decline in hip bone density after gastric bypass surgery is associated with extent of weight loss. *J Clin Endocrinol Metab* **93**, 3735-3740, doi:10.1210/jc.2008-0481 (2008).
233 De Prisco, C. & Levine, S. N. Metabolic bone disease after gastric bypass surgery for obesity. *Am J Med Sci* **329**, 57-61 (2005).
234 Von Mach, M. A. *et al.* Changes in bone mineral content after surgical treatment of morbid obesity. *Metabolism* **53**, 918-921 (2004).
235 Valderas, J. P. *et al.* Increase of bone resorption and the parathyroid hormone in postmenopausal women in the long-term after Roux-en-Y gastric bypass. *Obes Surg* **19**, 1132-1138, doi:10.1007/s11695-009-9890-y (2009).
236 Goode, L. R., Brolin, R. E., Chowdhury, H. A. & Shapses, S. A. Bone and gastric bypass surgery: effects of dietary calcium and vitamin D. *Obes Res* **12**, 40-47, doi:10.1038/oby.2004.7 (2004).
237 Soleymani, T., Tejavanija, S. & Morgan, S. Obesity, bariatric surgery, and bone. *Curr Opin Rheumatol* **23**, 396-405, doi:10.1097/BOR.0b013e328346f832 (2011).
238 Jin, J. *et al.* Increases in parathyroid hormone (PTH) after gastric bypass surgery appear to be of a secondary nature. *Surgery* **142**, 914-920; discussion 914-920 (2007).
239 Viegas, M., Vasconcelos, R. S., Neves, A. P., Diniz, E. T. & Bandeira, F. Bariatric surgery and bone metabolism: a systematic review. *Arq Bras Endocrinol Metabol* **54**, 158-163 (2010).
240 Johnson, J. M. *et al.* The long-term effects of gastric bypass on vitamin D metabolism. *Ann Surg* **243**, 701-704 (2006).
241 Goldner, W. S., O'Dorisio, T. M., Dillon, J. S. & Mason, E. E. Severe metabolic bone disease as a long-term complication of obesity surgery. *Obes Surg* **12**, 685-692, doi:10.1381/096089202321019693 (2002).
242 Slater, G. H. *et al.* Serum fat-soluble vitamin deficiency and abnormal calcium metabolism after malabsorptive bariatric surgery. *J Gastrointest Surg* **8**, 48-55; discussion 54-45 (2004).
243 Gasteyger, C. & Giusti, V. Nutritional follow-up after gastric bypass. *Rev Med Suisse* **2**, 844-847 (2006).
244 Vasconcelos, R. S. *et al.* Factors associated with secondary hyperparathyroidism in premenopausal women undergoing Roux-en-Y gastric bypass for the treatment of obesity. *Arq Bras Endocrinol Metabol* **54**, 233-238 (2010).
245 Signori, C., Zalesin, K. C., Franklin, B., Miller, W. L. & McCullough, P. A. Effect of gastric bypass on vitamin D and secondary hyperparathyroidism. *Obes Surg* **20**, 949-952, doi:10.1007/s11695-010-0178-z (2010).
246 Clements, R. H., Yellumahanthi, K., Wesley, M., Ballem, N. & Bland, K. I. Hyperparathyroidism and vitamin D deficiency after laparoscopic gastric bypass. *Am Surg* **74**, 469-474; discussion 474-465 (2008).
247 Levis, S. *et al.* Vitamin d deficiency and seasonal variation in an adult South Florida population. *J Clin Endocrinol Metab* **90**, 1557-1562, doi:10.1210/jc.2004-0746 (2005).
248 Hillebrand, J. J., Langhans, W. & Geary, N. Validation of computed tomographic estimates of intra-abdominal and subcutaneous adipose tissue in rats and mice. *Obesity (Silver Spring)* **18**, 848-853, doi:10.1038/oby.2009.341 (2010).
249 Abegg, K., Schiesser, M., Lutz, T. A. & Bueter, M. Acute peripheral GLP-1 receptor agonism or antagonism does not alter energy expenditure in rats after Roux-en-Y gastric bypass. *Physiol Behav*, doi:10.1016/j.physbeh.2013.03.027 (2013).
250 Saeidi, N. *et al.* Reprogramming of intestinal glucose metabolism and glycemic control in rats after gastric bypass. *Science* **341**, 406-410, doi:10.1126/science.1235103 (2013).
251 Dacks, P. A. & Rance, N. E. Effects of estradiol on the thermoneutral zone and core temperature in ovariectomized rats. *Endocrinology* **151**, 1187-1193, doi:10.1210/en.2009-1112 (2010).
252 Weir, J. B. New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol* **109**, 1-9 (1949).
253 Swift, R. W. The effect of feed on the critical temperature for the albino rat. *J Nutr*, 359-364 (1944).
254 Rossi, M. *et al.* Skin vasodilator function and vasomotion in patients with morbid obesity: effects of gastric bypass surgery. *Obes Surg* **21**, 87-94, doi:10.1007/s11695-010-0286-9 (2011).
255 Graessler, J. *et al.* Metagenomic sequencing of the human gut microbiome before and after bariatric surgery in obese patients with type 2 diabetes: correlation with inflammatory and metabolic parameters. *Pharmacogenomics J*, doi:10.1038/tpj.2012.43 (2012).
256 Kong, L. C. *et al.* Gut microbiota after gastric bypass in human obesity: increased richness and associations of bacterial genera with adipose tissue genes. *Am J Clin Nutr* **98**, 16-24, doi:10.3945/ajcn.113.058743 (2013).
257 Li, J. V. *et al.* Metabolic surgery profoundly influences gut microbial-host metabolic cross-talk. *Gut* **60**, 1214-1223, doi:10.1136/gut.2010.234708 (2011).

258 Zhang, H. *et al.* Human gut microbiota in obesity and after gastric bypass. *Proc Natl Acad Sci U S A* **106**, 2365-2370, doi:10.1073/pnas.0812600106 (2009).
259 Stemmer, K. *et al.* Roux-en-Y gastric bypass surgery but not vertical sleeve gastrectomy decreases bone mass in male rats. *Endocrinology* **154**, 2015-2024, doi:10.1210/en.2012-2130 (2013).
260 Sjogren, K. *et al.* The gut microbiota regulates bone mass in mice. *J Bone Miner Res* **27**, 1357-1367, doi:10.1002/jbmr.1588 (2012).
261 Asarian, L. *et al.* Estradiol increases body weight loss and gut-peptide satiation after Roux-en-Y gastric bypass in ovariectomized rats. *Gastroenterology* **143**, 325-327 e322, doi:10.1053/j.gastro.2012.05.008 (2012).
262 Krum, S. A. & Brown, M. Unraveling estrogen action in osteoporosis. *Cell Cycle* **7**, 1348-1352 (2008).
263 Manolagas, S. C., O'Brien, C. A. & Almeida, M. The role of estrogen and androgen receptors in bone health and disease. *Nat Rev Endocrinol*, doi:10.1038/nrendo.2013.179 (2013).
264 El-Kadre, L. J., Rocha, P. R., de Almeida Tinoco, A. C. & Tinoco, R. C. Calcium metabolism in pre- and postmenopausal morbidly obese women at baseline and after laparoscopic Roux-en-Y gastric bypass. *Obes Surg* **14**, 1062-1066, doi:10.1381/0960892041975505 (2004).
265 Vilarrasa, N. *et al.* Evaluation of bone mineral density loss in morbidly obese women after gastric bypass: 3-year follow-up. *Obes Surg* **21**, 465-472, doi:10.1007/s11695-010-0338-1 (2011).
266 Srivastava, S. *et al.* Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-kappa B ligand (RANKL)-induced JNK activation. *J Biol Chem* **276**, 8836-8840, doi:10.1074/jbc.M010764200 (2001).
267 Miyauchi, Y. *et al.* HIF1alpha is required for osteoclast activation by estrogen deficiency in postmenopausal osteoporosis. *Proc Natl Acad Sci U S A* **110**, 16568-16573, doi:10.1073/pnas.1308755110 (2013).
268 Melville, K. M. *et al.* Female mice lacking estrogen receptor-alpha in osteoblasts have compromised bone mass and strength. *J Bone Miner Res*, doi:10.1002/jbmr.2082 (2013).
269 Yang, Y. H. *et al.* Estradiol inhibits osteoblast apoptosis via promotion of autophagy through the ER-ERK-mTOR pathway. *Apoptosis*, doi:10.1007/s10495-013-0867-x (2013).
270 Gallagher, J. C., Riggs, B. L. & DeLuca, H. F. Effect of estrogen on calcium absorption and serum vitamin D metabolites in postmenopausal osteoporosis. *J Clin Endocrinol Metab* **51**, 1359-1364 (1980).
271 Takeda, S. *et al.* Leptin regulates bone formation via the sympathetic nervous system. *Cell* **111**, 305-317 (2002).
272 Wilms, B., Ernst, B., Schmid, S. M., Thurnheer, M. & Schultes, B. Enhanced Thermic Effect of Food After Roux-en-Y Gastric Bypass Surgery. *J Clin Endocrinol Metab* **98**, 3776-3784, doi:10.1210/jc.2013-1087 (2013).
273 Rubino, F. *et al.* The early effect of the Roux-en-Y gastric bypass on hormones involved in body weight regulation and glucose metabolism. *Ann Surg* **240**, 236-242 (2004).
274 Cornish, J., Callon, K. E., Cooper, G. J. & Reid, I. R. Amylin stimulates osteoblast proliferation and increases mineralized bone volume in adult mice. *Biochem Biophys Res Commun* **207**, 133-139, doi:10.1006/bbrc.1995.1163 (1995).
275 Dacquin, R. *et al.* Amylin inhibits bone resorption while the calcitonin receptor controls bone formation in vivo. *J Cell Biol* **164**, 509-514, doi:10.1083/jcb.200312135 (2004).
276 Cornish, J. *et al.* Effects of calcitonin, amylin, and calcitonin gene-related peptide on osteoclast development. *Bone* **29**, 162-168 (2001).
277 Yamada, C. *et al.* The murine glucagon-like peptide-1 receptor is essential for control of bone resorption. *Endocrinology* **149**, 574-579, doi:10.1210/en.2007-1292 (2008).
278 Zhong, Q. *et al.* Effects of glucose-dependent insulinotropic peptide on osteoclast function. *Am J Physiol Endocrinol Metab* **292**, E543-548, doi:10.1152/ajpendo.00364.2006 (2007).
279 Wong, I. P. *et al.* Peptide YY regulates bone remodeling in mice: a link between gut and skeletal biology. *PLoS One* **7**, e40038, doi:10.1371/journal.pone.0040038 (2012).
280 Kanazawa, I. Adiponectin in metabolic bone disease. *Curr Med Chem* **19**, 5481-5492 (2012).
281 Ahmad, N. N., Pfalzer, A. & Kaplan, L. M. Roux-en-Y gastric bypass normalizes the blunted postprandial bile acid excursion associated with obesity. *Int J Obes (Lond)*, doi:10.1038/ijo.2013.38 (2013).
282 Gerhard, G. S. *et al.* A role for fibroblast growth factor 19 and bile acids in diabetes remission after Roux-en-Y gastric bypass. *Diabetes Care* **36**, 1859-1864, doi:10.2337/dc12-2255 (2013).
283 Patti, M. E. *et al.* Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. *Obesity (Silver Spring)* **17**, 1671-1677, doi:10.1038/oby.2009.102 (2009).

284 Pournaras, D. J. *et al.* The role of bile after Roux-en-Y gastric bypass in promoting weight loss and improving glycaemic control. *Endocrinology* **153**, 3613-3619, doi:10.1210/en.2011-2145 (2012).
285 Werling, M. *et al.* Enhanced fasting and post-prandial plasma bile acid responses after Roux-en-Y gastric bypass surgery. *Scand J Gastroenterol*, doi:10.3109/00365521.2013.833647 (2013).
286 Laurenius, A. *et al.* Changes in eating behaviour and meal pattern following Roux-en-Y gastric bypass. *Int J Obes (Lond)* **36**, 348-355, doi:10.1038/ijo.2011.217 (2012).
287 Osto, M. *et al.* Roux-en-Y gastric bypass surgery in rats alters gut microbiota profile along the intestine. *Physiol Behav* **119**, 92-96, doi:10.1016/j.physbeh.2013.06.008 (2013).
288 Horowitz, M. *et al.* Effect of the once-daily human GLP-1 analogue liraglutide on appetite, energy intake, energy expenditure and gastric emptying in type 2 diabetes. *Diabetes Res Clin Pract* **97**, 258-266, doi:10.1016/j.diabres.2012.02.016 (2012).
289 Pannacciulli, N., Bunt, J. C., Koska, J., Bogardus, C. & Krakoff, J. Higher fasting plasma concentrations of glucagon-like peptide 1 are associated with higher resting energy expenditure and fat oxidation rates in humans. *Am J Clin Nutr* **84**, 556-560 (2006).
290 Barrera, J. G. *et al.* Hyperphagia and increased fat accumulation in two models of chronic CNS glucagon-like peptide-1 loss of function. *J Neurosci* **31**, 3904-3913, doi:10.1523/JNEUROSCI.2212-10.2011 (2011).
291 Nogueiras, R. *et al.* Direct control of peripheral lipid deposition by CNS GLP-1 receptor signaling is mediated by the sympathetic nervous system and blunted in diet-induced obesity. *J Neurosci* **29**, 5916-5925, doi:10.1523/JNEUROSCI.5977-08.2009 (2009).
292 Kanoski, S. E., Fortin, S. M., Arnold, M., Grill, H. J. & Hayes, M. R. Peripheral and central GLP-1 receptor populations mediate the anorectic effects of peripherally administered GLP-1 receptor agonists, liraglutide and exendin-4. *Endocrinology* **152**, 3103-3112, doi:10.1210/en.2011-0174 (2011).
293 Ockenga, J. *et al.* Plasma bile acids are associated with energy expenditure and thyroid function in humans. *J Clin Endocrinol Metab* **97**, 535-542, doi:10.1210/jc.2011-2329 (2012).
294 Watanabe, M. *et al.* Lowering bile acid pool size with a synthetic farnesoid X receptor (FXR) agonist induces obesity and diabetes through reduced energy expenditure. *J Biol Chem* **286**, 26913-26920, doi:10.1074/jbc.M111.248203 (2011).
295 Watanabe, M. *et al.* Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* **439**, 484-489, doi:10.1038/nature04330 (2006).
296 Saeidi, N. *et al.* Sleeve gastrectomy and Roux-en-Y gastric bypass exhibit differential effects on food preferences, nutrient absorption and energy expenditure in obese rats. *Int J Obes (Lond)* **36**, 1396-1402, doi:10.1038/ijo.2012.167 (2012).
297 Stefater, M. A. *et al.* Sleeve gastrectomy induces loss of weight and fat mass in obese rats, but does not affect leptin sensitivity. *Gastroenterology* **138**, 2426-2436, 2436 e2421-2423 (2010).
298 Faria, S. L., Faria, O. P., Cardeal Mde, A., de Gouvea, H. R. & Buffington, C. Diet-induced thermogenesis and respiratory quotient after Roux-en-Y gastric bypass. *Surg Obes Relat Dis* **8**, 797-802, doi:10.1016/j.soard.2012.06.008 (2012).
299 Werling, M. *et al.* Increased postprandial energy expenditure may explain superior long term weight loss after Roux-en-Y gastric bypass compared to vertical banded gastroplasty. *PLoS One* **8**, e60280, doi:10.1371/journal.pone.0060280 (2013).
300 Gao, Z. *et al.* Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes* **58**, 1509-1517, doi:10.2337/db08-1637 (2009).
301 Confavreux, C. B. Bone: from a reservoir of minerals to a regulator of energy metabolism. *Kidney Int Suppl*, S14-19, doi:10.1038/ki.2011.25 (2011).
302 Ducy, P. The role of osteocalcin in the endocrine cross-talk between bone remodelling and energy metabolism. *Diabetologia* **54**, 1291-1297, doi:10.1007/s00125-011-2155-z (2011).
303 Fernandez-Real, J. M. & Ricart, W. Osteocalcin: a new link between bone and energy metabolism. Some evolutionary clues. *Curr Opin Clin Nutr Metab Care* **14**, 360-366, doi:10.1097/MCO.0b013e328346df4e (2011).
304 Ferron, M. *et al.* Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. *Cell* **142**, 296-308, doi:10.1016/j.cell.2010.06.003 (2010).
305 Khor, E. C., Wee, N. K. & Baldock, P. A. Influence of hormonal appetite and energy regulators on bone. *Curr Osteoporos Rep* **11**, 194-202, doi:10.1007/s11914-013-0157-0 (2013).
306 Modica, S. & Wolfrum, C. Bone morphogenic proteins signaling in adipogenesis and energy homeostasis. *Biochim Biophys Acta* **1831**, 915-923, doi:10.1016/j.bbalip.2013.01.010 (2013).
307 Movahed, A. *et al.* Reduced serum osteocalcin concentrations are associated with type 2 diabetes mellitus and the metabolic syndrome components in postmenopausal women: the crosstalk between bone and energy metabolism. *J Bone Miner Metab* **30**, 683-691, doi:10.1007/s00774-012-0367-z (2012).

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